Volume 3 *Number 3* *June 1957*

CLINICAL CHEMISTRY

Journal of the American Association of Clinical Chemists

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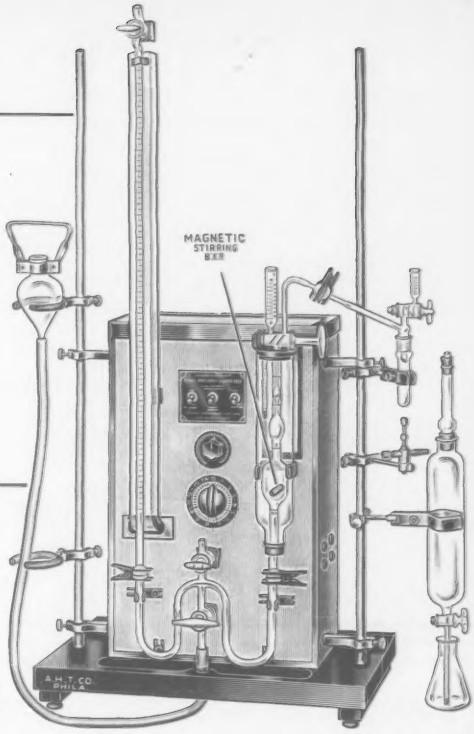

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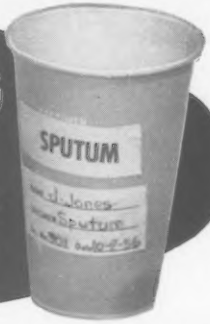

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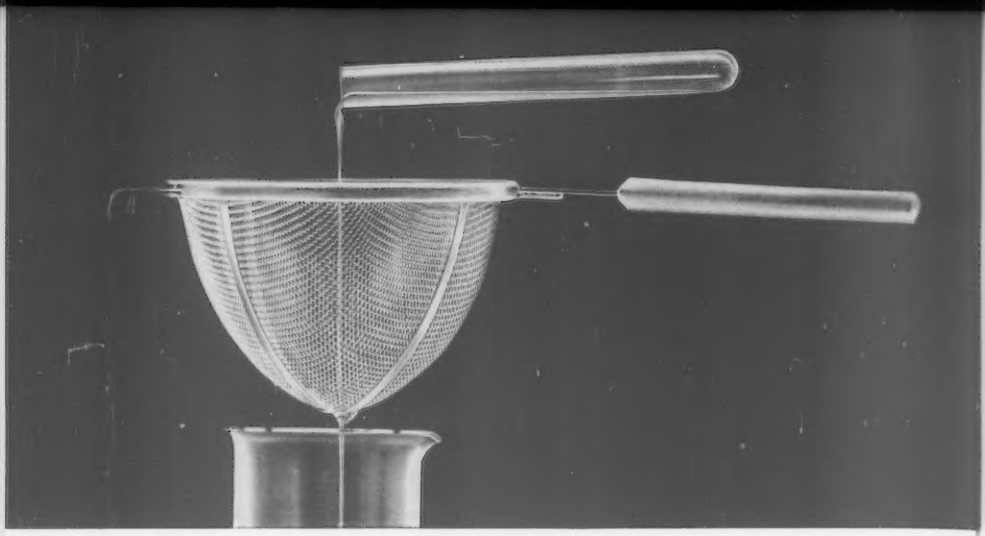

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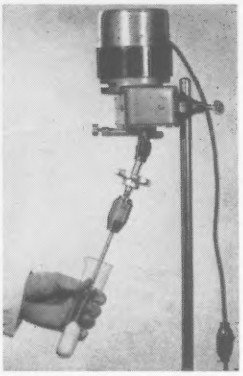

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Advertiser	*Page*
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Chem-Tech Laboratories	xii
Clay-Adams, Inc.	iii
Coleman Instruments, Inc.	vii
Delta Chemical Works, Inc.	viii
Distillation Products Industries	x
Eberbach Corporation	viii
Fisher Scientific Company	xvi
Hartman-Leddon Company	3rd Cover
Hyland Laboratories	v
International Equipment Co.	xiii
Meinecke & Company, Inc.	x
Nuclear-Chicago Corporation	2nd Cover
Photovolt Corporation	xii
Professional Tape Company, Inc.	v
The Samson Laboratories	viii
Scientific Products	xi
Standard Scientific	4th Cover
Arthur H. Thomas Company	i
Warner-Chilcott Laboratories	xiv

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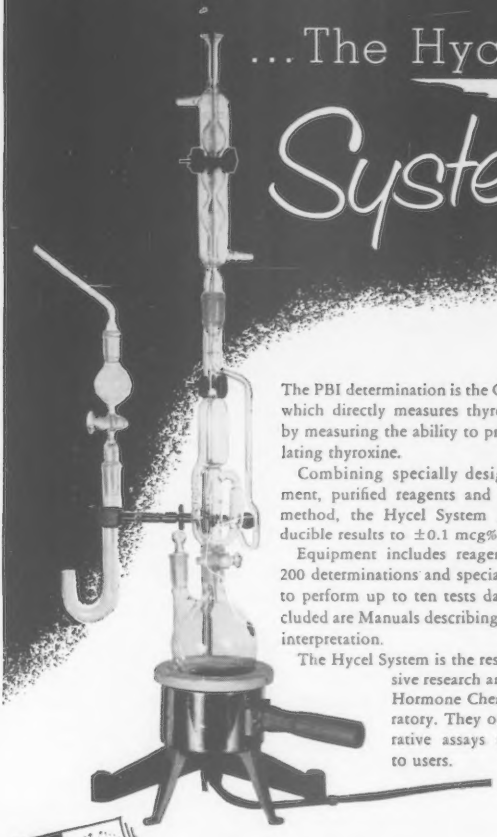

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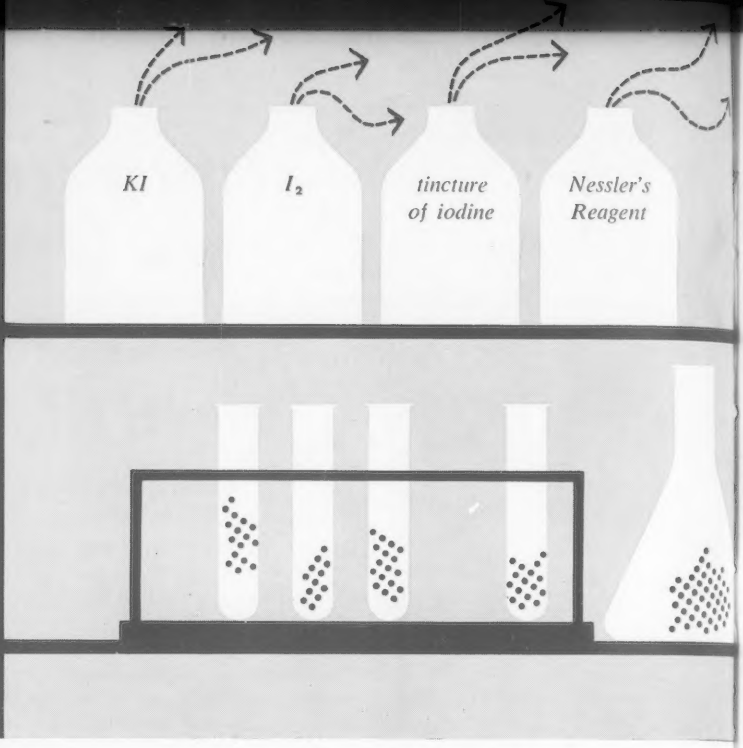

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CONTENTS

Chemical Findings in Patients with Acute Nonfatal Barbiturate Intoxication *Irving Sunshine and Earl Hackett* 125

New Tests for Bile and Detection of Bile in Serum and Urine *M. Z. Barakat, S. K. Shehab, and M. M. El-Sadr* 135

Urobilinogen Excretion in Normal Adults: Results of Assays with Notes on Methodology *Bernard Balikov* 145

Paper Chromatography of Free Amino Acids in Human Blood Serum *Vladimír Jirgl* 154

Comparison of Plasma Uric Acid Levels Obtained with Five Different Methods *Eugene L. Kanabrocki, Joseph Greco, Lee Wilkoff, and Roger Veatch* 156

Interference of Bromide in the Zak Ferric Chloride-Sulfuric Acid Cholesterol Method, and Means of Eliminating This Interference *Eugene W. Rice and Dolores B. Lukasiewicz* 160

Simple Specific Test for Urine Glucose *Alfred H. Free, Ernest C. Adams, Mary Lou Kercher, Helen M. Free, and Marion H. Cook* 163

Measurement of Uric Acid in Biologic Fluids Using an Ion-Exchange Separation *Bernard Shapiro, David Seligson, and Ralph Jessar* 169

Total Neutral 17-Ketosteroids: Clinical Method for Measurement *Marian S. Kafka and Philip K. Bondy* 178

Photometric Procedure for Determining Esterase Activity *Harold A. Ramsey* 185

Paper Electrophoresis of Serum Proteins: A Note *L. D. Mellor* 195

The Clinical Chemist 196

Book Reviews 201

Abstracts 202

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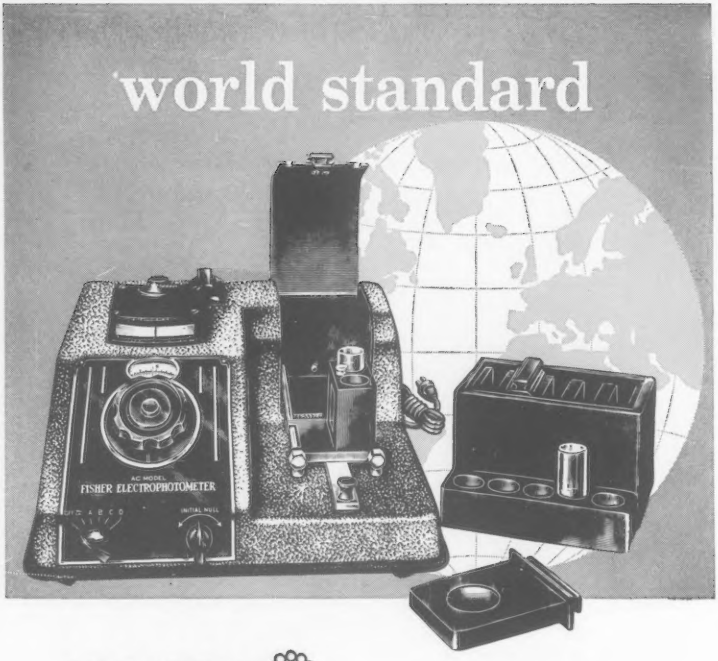
September 9-13, 1957, New York, N. Y.



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B-19b

Chemical Findings in Patients with Acute Nonfatal Barbiturate Intoxication

Irving Sunshine and Earl Hackett

A RECENT REPORT from this laboratory (1) disclosed that in the metropolitan area of Cleveland barbiturate compounds are responsible for approximately one-third of all toxic reactions of sufficient severity to require hospitalization. That this situation is not unique to this community is indicated by many recent reports from other localities in the United States as well as from abroad (2–7).

Inasmuch as the victim of barbiturate poisoning is usually unconscious when first seen by a physician, recognition of the cause and severity of the intoxication depends largely on chemical identification and quantitation of the particular compound involved. Ultraviolet spectrophotometric methods developed by Goldbaum (8), Broughton (5), Wright (4), Walker (9), and others (10, 11) permit rapid and reliable quantitative identification of barbiturate compounds in the blood and urine.

Studies of nonfatal barbiturate intoxications (2–5, 8–18) have only reported the initial blood barbiturate levels found at the time of the patient's admission to the hospital. In only some few cases (4, 5, 12, 18) are several successive blood barbiturate levels reported from the same hospitalized patient. These studies indicate that there probably is a direct correlation between the patient's clinical condition and the blood barbiturate level. This study presents additional data to support this thesis.

MATERIAL AND METHODS

The data presented in this study were obtained from 44 patients who were admitted to several hospitals in this vicinity because of barbiturate intoxication. Samples of blood and urine were taken periodically for analysis. When urine samples were available, efforts were made to collect

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them in such a manner that renal clearances could be calculated. Each time that samples were taken for chemical analysis the clinical state of the patient was noted and classified in one of the following five categories:

Stage I. Awake, competent, and mildly sedated.

Stage II. Sedated, reflexes present, prefers sleep, answers questions when roused, does not cerebrate properly.

Stage III. Comatose, reflexes present.

Stage IV. Comatose, no reflexes obtainable.

Stage V. Comatose, no reflexes obtainable, and with circulatory and/or respiratory difficulty.

These stages do not have definite limits, but blend into each other. The deep tendon reflexes were used as the criteria for the presence of reflexes. Corneal, pupillary, and abdominal responses are subject to more variability in relation to the levels of anesthesia. Gag and cough reflexes return at about the same time as the deep tendon reflexes; response to pain returns later. The respiratory difficulty in Stage V is due to direct depression of the medullary respiratory center and is not due to the secondary pneumonia which often develops in these patients. All barbiturates were qualitatively and quantitatively determined, using a slight modification of Goldbaum's technic (8). The modification is in the adjustment of the 0.45N NaOH extract of the barbiturate to *p*H 10.5. This is accomplished by adding 0.5 ml. of 16% NH_4Cl to the 3 ml. of 0.45N NaOH in the Beckman cuvet. The resulting mixture has the required *p*H and can then be read directly (19). Duplicate determinations using this technic checked within 10 per cent. All barbiturate levels are reported as the sodium salts of the respective barbituratic acid.

Whenever possible, the qualitative identification of the barbiturate derivative was confirmed by melting-point determinations, micro crystalline appearances or, more recently, paper chromatographic technics (20–22).

Since the amount of drug taken and the time of ingestion could only be ascertained by anamnestic data of questionable reliability, these data are not presented in this study. Consequently, all times mentioned throughout the discussion are based on the period between a given observation and the moment the patient was admitted to the hospital.

RESULTS

A previous paper by the authors (18) presented some data on the correlation of a patient's clinical condition following phenobarbital ingestion with his blood phenobarbital level. The phenobarbital data are supple-

mented and included in the current study (Tables 1–4). The number of patients studied is large enough to make conclusions from the data statistically reliable. Table 1 corroborates the authors' previous findings with respect to phenobarbital intoxication. Data by other investigators (2–5) also agree that recovery from phenobarbital intoxication occurs when the blood phenobarbital level is 4.0–5.5 mg. per 100 ml.

Stage II, the ability of the patient to respond to questions, even if lethargic and sleepy, is the criterion of recovery that is used throughout this discussion.

Table 1. Blood Phenobarbital Levels[a] with Respect to Patients' Clinical Condition (mg./100 ml.)

	Stage				
Case	*V*	*IV*	*III*	*II*	*I*
B. S.	..	..	..	4.0	2.4
I. B.	..	..	3.8	..	3.3
C. H.	..	..	..	4.3	..
M. A.	..	..	..	4.6	3.6
G. W.	..	..	7.7	..	..
R. H.	..	..	6.1	..	..
W. Z.	..	11.0	..	..	..
D. W.	..	..	7.4	..	3.9
T. L.	..	..	..	..	2.0
A. Z.	..	..	..	..	1.2
P. P.	20.6	9.2	..	..	..
S. S.	..	9.2	5.0	4.2	3.5
E. G.	..	..	6.1	..	..
J. D.	..	..	..	..	2.2

[a] The blood levels represent that point where the patients entered a given clinical stage.

Table 2. Blood Amobarbital Levels[a] with Respect to Patients' Clinical Condition (mg./100 ml.)

	Stage				
Case	*V*	*IV*	*III*	*II*	*I*
A. W.	..	5.6	3.5	..	..
J. L.	7.9	6.2	5.8	2.0	1.4
J. D.	..	4.2	2.5	2.2	1.5
A. G.	..	6.2	4.1	..	..
M. C.	8.6	5.6	4.7	2.6	..
R. H.	..	5.1	..	..	..
K. G.	8.9	..	..	..	..
H. K.	..	..	3.0	..	..

[a] The blood levels represent that point where the patients entered a given clinical stage.

Table 3. Blood Pentobarbital Levels[a] with Respect to Patients' Clinical Condition (mg./100 ml.)

	Stage				
Case	*V*	*IV*	*III*	*II*	*I*
F. G.	3.5	2.7	2.2	..	0.6
F. R.	..	..	3.4	..	..
J. B.	..	..	..	1.1	..
A. H.	..	..	..	1.7	..
D. R.	..	..	..	1.5	..
S. S.	..	..	1.5	1.2	..
M. B.	..	4.2	3.5	2.6	1.8
D. F.	..	..	2.5	1.7	..
L. W.	..	..	3.4	1.7	..
H. W.	..	..	..	2.2	..
C. B.	..	..	3.3	..	..
T. A.	..	..	2.5	..	..

[a] The blood levels represent that point where the patients entered a given clinical stage.

Table 4. Blood Secobarbital Levels[a] with Respect to Patients' Clinical Condition (mg./100 ml.)

	Stage				
Case	*V*	*IV*	*III*	*II*	*I*
A. H.	..	..	2.3	1.2	0.6
J. D.	..	..	1.1	..	..
A. I.	..	3.5	2.4	..	..
C. G.	..	4.6	2.8	1.7	1.0
E. A.	..	..	..	..	0.6
R. W.	..	..	2.9	1.7	..
E. K.	..	..	..	..	1.0
L. W.	..	..	..	..	0.6
M. A.	..	..	..	..	1.2
L. S.	..	..	..	1.7	1.1

[a] The blood levels represent that point where the patients entered a given clinical stage.

Table 2 presents the findings in cases of amobarbital intoxication. The blood amobarbital level at which recovery was noted was in the range of 2.0–2.5 mg. per 100 ml. Severe intoxication was always noted if the blood amobarbital level was higher than 5.5–6.0 mg. per 100 ml., the recovery level for many phenobarbital-intoxicated patients. This serves to emphasize the need for qualitative, as well as quantitative, chemical analysis in all cases of barbiturate intoxication. In any given individual, the severity of the intoxication is a function, among other factors, of not only how much, but also, which barbiturate compound was ingested.

This thesis is further substantiated by the data in Tables 3 and 4. In the cases of pentobarbital and secobarbital, patients recovered with blood barbiturate levels ranging from 1.5–2.5 mg. per 100 ml. and 1.2–1.7 mg. per 100 ml., respectively. Severe intoxication was noted whenever the blood barbiturate levels were higher than 3.5 mg. per 100 ml. It is interesting to note that according to their respective blood barbiturate levels, 3 patients, M. B. in Table 3 and C. G. and R. W. in Table 4, were more sedated than the average. Anomalous results might be expected in any individual as a result of tolerance, a pathologic condition, or the presence of other chemical agents. In these three cases, the atypical data are probably due to the simultaneous presence of ethyl alcohol. This synergism between ethanol and the barbiturates has been documented previously (23, 24).

A summary of all the data on the blood barbiturate level as a function

Table 5. Average Barbiturate Levels with Respect to Patients' Clinical Condition (mg./100 ml.)

	Stage				
	V	*IV*	*III*	*II*	*I*
Phenobarbital	..	10.0	6.5	4.4	3.5
Amobarbital	8.6	6.2	4.7	2.5	1.5
Secobarbital	..	..	2.5	1.5	0.8
Pentobarbital	..	..	3.0	2.0	..

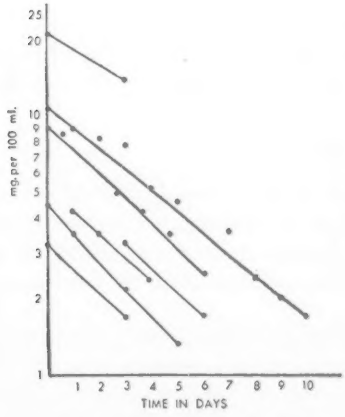

Fig. 1. Blood phenobarbital level vs. time after hospital admission.

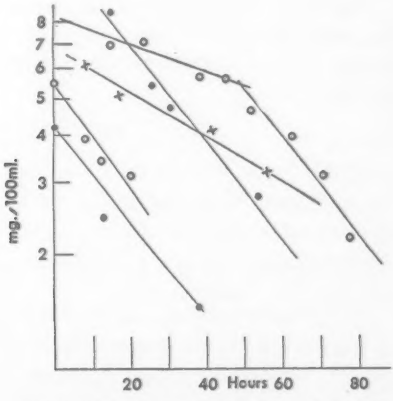

Fig. 2. Blood Amytal level vs. time after hospital admission.

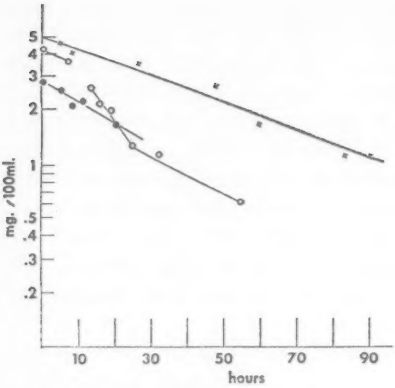

Fig. 3. Blood secobarbital level vs. time after hospital admission.

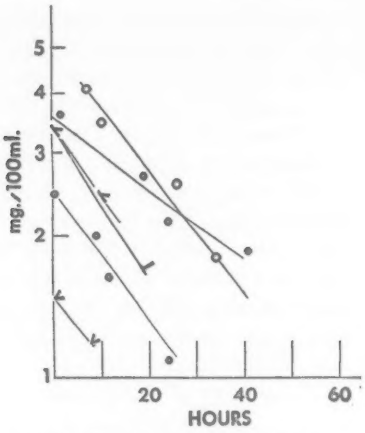

Fig. 4. Blood pentobarbital level vs. time after hospital admission.

of a patient's clinical condition is presented in Table 5. The data obtained by other investigators in this field are consistent with those in the summary.

Chemical analyses are being used more frequently to confirm the clinical diagnosis of barbiturate intoxication. Whenever a positive chemical analytical result is obtained, the invariable question to be answered is how long it will take for the patient to recover from his barbiturate intoxication, presuming no other complications supervene.

Figures 1–4 help answer this question. Since the data are linear on the semilogarithmic coordinate system, the decrease in blood barbiturate concentration is exponentially related to the time elapsing following ingestion.

Half-Life of Barbiturates

On the basis of these data, the half-life of the several barbiturates can be calculated. (The half-life is that period of time required for the blood level to fall to half its original value.) Another instructive calculation is the percentage fall in a given blood level in one day. Both the results are presented in Table 6.

As one would expect, the half-life and the percentage of daily decrease of the barbiturate level are a direct function of the duration of action of these agents as recognized clinically. The longer-acting barbiturates have a longer half-life and lower percentage of daily decrease than do the shorter-acting barbiturates. Lous presented similar data on the daily

Table 6. Rate of Disappearance of Barbiturates from Blood

	Half-life	*Daily % decrease*
Phenobarbital	72 hr.	21
Amobarbital	25 hr.	48
Pentobarbital	20 hr.	57

blood barbiturate decrease: from 10–15 per cent for barbital, 14–27 per cent for phenobarbital, and 35–40 per cent for aprobarbital (Alurate) (2, 15).

Urine barbiturate levels are frequently requested by clinicians as a diagnostic aid. Urinary barbiturate analysis indicates only that a barbiturate is being excreted and is not an indication of the degree of his central nervous system depression. It may help in the qualitative identification of the barbiturate implicated. However, this is all one can or should expect from such a determination.

Renal Clearances

Since renal excretion is one way the body disposes of chemical agents, data were accumulated on the urinary excretion of barbiturates. Figures 5–7 show the renal clearances of barbiturates as a function of the rate of urine flow. These data indicate that the clearance is greater at higher urine flows. No leveling off of the curves was noted at higher urine flows.

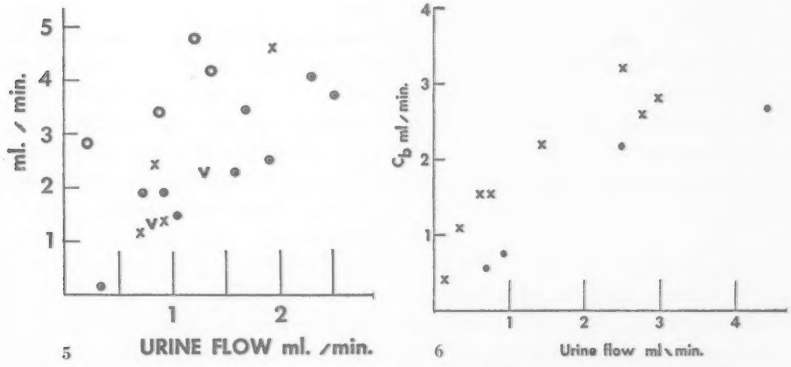

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Fig. 5. Renal clearance of Amytal vs. urine flow. Each set of symbols represents different renal clearances obtained from the same patient at different periods during his hospital stay. **Fig. 6.** Renal clearance of Seconal vs. urine flow. Each set of symbols represents different renal clearances obtained from the same patient at different periods during his hospital stay.

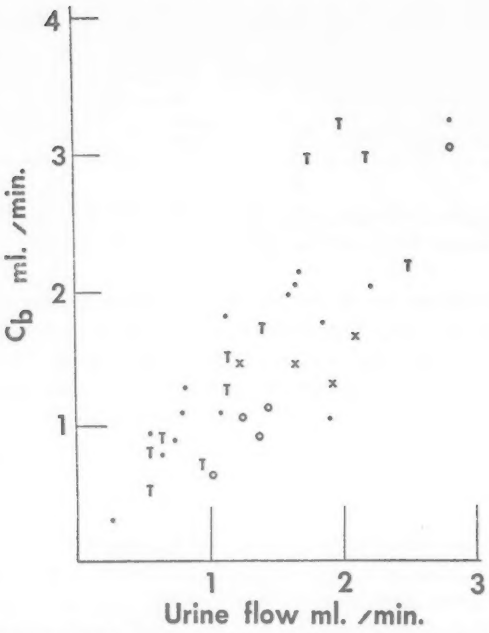

Fig. 7. Renal clearance of phenobarbital vs. urine flow. Each set of symbols represents different renal clearances obtained from the same patient at different periods during his hospital stay.

Lous (2) studied renal clearances of phenobarbital and barbital. He found that these renal clearances increased from two to four times as the urine flow rate is raised from 0.5 ml./min. to 4 ml./min. No appreciably further rise in clearance values were noted at higher urine flow rates. The data in this study confirm Lous' first conclusion. No urine flow rates higher than 4 ml./min., were attained in any patient in this study. No comment can be made, therefore, on Lous' second conclusion.

Total Barbiturate Excretion

The total amount of barbiturates excreted daily in the urine was never very high. Actual amounts varied from 50 to 60 mg./day in the short-acting barbiturates to 180 mg./day for the longer-acting ones. Only in the case of barbital did this urine excretion seem significant. In the one case studied, 6 Gm. were excreted in a 5-day period! The other data obtained in this one barbital case are recorded in Table 7.

Table 7. Chemical and Clinical Study of A Case of Acute Barbital Intoxication

Time (days)	*Blood level* (mg./100 ml.)	*Clinical stage*	*Urine*			*Renal clearance* (ml./min.)
			Conc. (mg./100 ml.)	*Vol.* (ml.)	*Daily excretion* (mg.)	
0	24.7	V	1	..	..	..
1	20.9	V	176.	450	792	4.95
2	17.2	IV	93.	770	716	5.2
3	9.0	III	62.	4246	2604	13.9
4	6.6	II	56.5	2770	1250	11.4
5	5.0	I	41.	1600	656	7.95

DISCUSSION

The data presented in Tables 1–4 indicate that there is a reasonably acceptable correlation between a patient's clinical condition and his blood barbiturate level. There is a sufficiently small variation in each range so that the data in Table 5 represent average values that one can use to interpret the results of a single quantitative chemical analysis.

In general, a patient with a blood phenobarbital level of less than 6.0 mg. per 100 ml., a blood amobarbital level of less than 4.5 mg. per 100 ml., or a blood pentobarbital or secobarbital level of less than 2.5 mg. per 100 ml. will not be seriously ill due to the barbiturate alone. Although he may be comatose, reasonably conservative therapy (25) is all this patient requires.

Potentiation of the effects of the barbiturate by other drugs may occasionally confuse the clinical picture. The authors have seen several cases of concurrent ingestion of ethanol and a barbiturate and one case of simultaneous chlorpromazine-barbiturate ingestion. In all these cases, the patients were much more depressed than one would expect from the barbiturate alone. When a patient appears more depressed than would be expected from the blood barbiturate level, one should always investigate the possibility of concurrent intoxication with other chemical agents. Trauma may also be a factor in some of these atypical cases. The presence of intracranial lesions will deepen the depression due to a given dose of a barbiturate. However, physical examination will usually reveal the presence of these conditions.

Tolerance will also give atypical data. The authors have discussed the problem in more detail elsewhere (26). They presented eight cases in which evidence was found, clinically and chemically, of tolerance to phenobarbital. Such patients, with blood phenobarbital levels of 7–8 mg. per 100 ml. were competent and awake. Isbell (27), Lous (11), and

Wright (4) have also given examples of tolerance. Brodie (28) has suggested the presence of acute tolerance due to Pentothal administration, but other investigators have found an acute tolerance does not appear to occur with the other barbiturates.

SUMMARY

1. Clinical and chemical data on acutely intoxicated patients show a direct quantitative relationship of the patient's blood barbiturate levels to his clinical condition.

2. The "half-life" of phenobarbital, amobarbital, and pentobarbital was determined to aid in prognosis of an intoxicated patient with a given blood barbiturate level.

3. Urinary clearance studies showed that the clearance was directly proportional to the urine flow.

4. A patient's urine barbiturate level has no relation to his clinical condition.

REFERENCES

1. Sunshine, I., and Adelson, L., *J. Criminal Law, Criminol. and Police Sci.* **44,** 116 (1953).
2. Lous, P., *Acta Pharmacol. Toxicol.* **10,** 147 (1954).
3. Copeman, P. R. vander, *J. Forensic Med.* **1,** 271 (1954).
4. Wright, J. P. T., *Quart. J. Med. (Oxford)* **24,** 95 (1955).
5. Broughton, P. M. S., Higgins, G., and O'Brien, J. R. P., *Lancet* **270,** 180 (1956).
6. Moore, M., Alexander, L., and Ipson, I., *New Eng. J. Med.* **246,** 46 (1952).
7. Nillson, E., *Acta Med. Scand.* **Suppl.** 253 (1951).
8. Goldbaum, L., *Anal. Chem.*, **24,** 1604 (1952).
9. Walker, J. T., Fisher, R. S., and McHugh, J. J., *Amer. J. Clin. Path.* **18,** 451 (1948).
10. Gould, T. C., and Hine, C. H., *J. Lab. Clin. Med.* **34,** 1462 (1949).
11. Lous, P., *Acta Pharmacol. Toxicol.* **6,** 227 (1950).
12. Lous, P., Ibid **10,** 166 (1954).
13. Wright, J. T., and Johns, R. G. S., *J. Clin. Path.* **6,** 78 (1953).
14. Fisher, R. S., Walker, J. T., and Plummer, C. W., *Amer. J. Clin. Path.* **18,** 462 (1948).
15. Lous, P., *Acta Pharmacol. Toxicol.* **10,** 261 (1954).
16. Reed, C. E., Driggs, M. F., and Foot, C. C., *Ann. Int. Med.* **37,** 290 (1952).
17. Roche, M., Wynne, L. C., and Haskins, D. M., Ibid **33,** 73 (1950).
18. Sunshine, I., and Hackett E., *Amer. J. Clin. Path.* **24,** 1133 (1954).
19. Abernathy, R., Personal communication.
20. Algeri, E., and McBay, A., *Amer. J. Clin. Path.* **22,** 37 (1952).
21. Grieg, *Nature* **170,** 845 (1952).
22. Curry, A. S., *J. Pharm. and Pharmacol.* **7,** 1072 (1955).
23. Ramsay H., and Haag, H. B., *J. Pharm. Exper. Ther.* **88,** 313 (1946).
24. Jetter, W. W., and McLean, R., *Arch. Path.* **36,** 112 (1943).
25. Echenhoff, J. E., and Dam, W., *Amer. J. Med.* **20,** 912 (1956).
26. Sunshine, I., and Hackett, E., *J. Lab. Clin. Med.* (in press).
27. Isbell, G., *Arch. Neur. et Psych.* **64,** 1 (1950).
28. Brodie, B. B., *Fed. Proc.* **11,** 632 (1952).

New Tests for Bile and Detection of Bile in Serum and Urine

M. Z. Barakat, S. K. Shehab, and M. M. El-Sadr

THE PURPOSES OF THE PRESENT PAPER are to describe some new sensitive tests suitable for the detection of bile in biologic fluids which overcome the defects demonstrated in procedures described in most standard texts, and also to confirm the experiments of C. E. May *et al.* (1). The sensitivity of the proposed oxidation tests to detect bilirubin, *viz.* ferricyanide, molybdate, *N*-bromosuccinimide (2, 3), *N*-chlorosuccinimide (4), and dichromate tests, is compared with the previously known Gmelin, Fouchet, Obermeyer, Nakayama, iodine and nitrite tests. Similarly a comparison of the proposed diazo tests, i.e., diazochlorides obtained from various members of sulfonamides, as well as from the aromatic amines previously tested by May *et al.* (1), with the previously known diazotized sulfanilic acid test is reported.

MATERIALS

1. Potassium ferricyanide reagent: To 12.5 Gm. of trichloroacetic acid in 50 ml. of distilled water add 5 ml. of 10 per cent (w/v) potassium ferricyanide aqueous solution and mix well. The reagent is kept in a dark bottle.
2. Saturated aqueous ammonium molybdate solution.
3. 0.1% (w/v) *N*-bromosuccinimide aqueous solution.
4. 0.1% (w/v) *N*-chlorosuccinimide aqueous solution.
5. 7% (w/v) aqueous potassium dichromate solution.
6. Stock Solutions:

Diazo I: 0.1 Gm. of the aromatic amino compound, e.g., anthranilic acid, etc., was dissolved in 1.5 ml. of concentrated HCl and some

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distilled water or 20 ml. of alcohol in the case of α- or β-naphthylamine and diluted with distilled water to 100 ml. This solution should be prepared fresh daily or refrigerated at 4° for storage.

Diazo II: 0.5% (w/v) aqueous sodium nitrite solution. *Diazo Reagent* was freshly prepared as follows:

To 25 ml. of Diazo I, add 0.75 ml. of Diazo II and mix well. The diazo reagent must be kept in a dark bottle, especially in the case of α-naphthylamine diazo reagent which becomes a strong purple on standing exposed to light.

7. Bilirubin Solution is prepared by dissolving 5 mg. of bilirubin in cold, neutral alcohol, then add 2 ml. of 2% (w/v) NaOH aqueous solution and diluted with neutral alcohol to 100 ml. One milliliter of this solution contains 50 μg. of bilirubin.

8. The previously known Fouchet's, Nakayama's, and Obermeyer's reagents have been prepared according to the literature.

METHODS AND EXPERIMENTAL RESULTS

Experiments on Bilirubin Test Solutions

Oxidation Tests

Starting with 3 ml. of the bilirubin solution, i.e., containing 150 μg. of bilirubin, various dilutions were prepared by mixing 2, 1, 0.8, 0.6, 0.4, 0.2, and 0.1 ml. of the bilirubin solution with 1, 2, 2.2, 2.4, 2.6, 2.8, and 2.9 ml. of distilled water, respectively, to obtain the various concentrations of bilirubin in 3 ml. solution (Table 1), on which the proposed oxidation tests were carried out at the same time with those previously known.

Table 1. Comparative Sensitivity of the New Oxidation Tests with Some Previous Tests

Test	*Bilirubin content in μg./3 ml. of solution*				
	40	*30*	*20*	*10*	*5*
Gmelin's	−	−	−	−	−
Fouchet's	+	+	Trace	−	−
Obermeyer's	+	+	Trace	−	−
Nakayama's	+	+	Trace	−	−
Potassium ferricyanide	+	+	Trace	−	−
Ammonium molybdate	+	+	+	−	−
N-Bromosuccinimide	+	+	+	−	−
N-Chlorosuccinimide	+	+	+	−	−
Potassium dichromate	+	+	+	+	+

All tests gave positive results on bilirubin solutions containing 50 μg./3 ml. or higher.

Table 2. Comparison of the Sensitivity of the New Coupling Tests with Ehrlich's Diazo Test

Diazo reagent of	*No. drops of diazo reagent*	*Bilirubin content in μg./ml. of solution*		
		15	*10*	*5*
Sulfanilic acid	4	+	Trace	–
Anthranilic acid	2	+	–	–
Benzidine	2	+	–	–
p-Toluidine	2	+	Trace	–
Sulfaguanidine	5	+	Trace	Trace
Aniline	2	+	+	Trace
p-Nitroaniline	5	+	+	Trace
p-Aminobenzoic acid	3	+	+	–
α- or β-Naphthylamine	5	+	+	–
Sulfanilamide	3	+	+	–
Sulfathiazole	4	+	+	Trace
Sulfadiazine	5	+	+	Trace

All tests were positive when bilirubin concentration was 15 μg./ml. or higher.

Diazo Tests

Coupling tests for bilirubin were carried out by adding 2 to 5 drops of the diazo reagent to 1 ml. of the bilirubin solution, i.e., containing 50 μg. of bilirubin or of the various dilutions such as those obtained by mixing 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1 ml. of the bilirubin solution with 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 ml. of distilled water, respectively (Table 2). A purple-violet color was formed with the diazo reagents investigated.

The previously tested diazochlorides prepared from, e.g., aniline, *o*- and *p*-toluidine, benzidine, etc., did not give any color when bilirubin was dissolved in neutral alcohol; the purple-violet color appeared only on the addition of one drop of 2% (w/v) NaOH.

In the case of low concentrations of bilirubin, starting from 15 μg./ml., one or two drops of the diazo reagent were only added to detect bilirubin.

The blank test was always colorless or pale yellow even upon addition of 2 drops of 2% NaOH, except in the case of the α-naphthylamine diazo reagent. When 1 ml. of neutral alcohol was treated with 5 drops of this latter diazo reagent, a faint purple color appeared.

Experiments on Bile

The following new tests have been applied on fresh ox bile.

Tests for Bile Pigments

I. (a) To 5 ml. of diluted bile (5%) in a test tube add 2 ml. of the potassium ferricyanide reagent, a bluish-green color appears.

(b) To 5 ml. of the diluted bile add 0.5 ml. of 10% potassium ferricyanide solution and add 3 ml. of conc. H_2SO_4 down the side of the test tube. At the point of contact green and red rings appear. A blank shows no colored rings.

II. To 5 ml. of the diluted bile add 2 ml. of saturated ammonium molybdate solution and then 3 ml. of conc. H_2SO_4 added down the side of the test tube; a blue ring appears at the interface of the two liquids. On shaking, the solution assumes a blue color. A control, using water alone, is colorless.

III. To 5 ml. of the diluted bile add 2 drops of 0.1% (w/v) *N*-bromosuccinimide or *N*-chlorosuccinimide aqueous solution; a blue color is produced. A blank is colorless.

IV. To 5 ml. of the diluted bile add 0.5 ml. of 7% potassium dichromate solution, then carefully add 3 ml. of conc. H_2SO_4 so that the two fluids do not mix. At the point of contact a blue ring appears. A blank, using water alone, shows an orange-red color.

V. To 5 ml. of the diluted bile, add 2 ml. of freshly prepared diazo reagent of any of the following aromatic amino compounds: aniline, *p*-toluidine, anthranilic acid, etc. In this test the compound azobilirubin is formed either directly or after the addition of 2 to 4 drops of 2% NaOH as indicated in the following table.

Diazo Reagent of	*2% NaOH*	*Color*
p-Aminobenzoic acid	−	Purple-violet
p-Nitroaniline	−	Pink
Sulfadiazine, Sulfaguanidine, Sulfathiazole, Sulfanilamide	−	Purple-violet
Aniline	+	Purple
p-Toluidine	+	Purple
α- or β-Naphthylamine	+	Pink
Anthranilic acid	+	Purple

A blank using water instead of bile solution, is either colorless or very faint purple, e.g., in the case of the diazo reagent prepared from *p*-toluidine or α- and β-naphthylamines.

Interfering Substances

Various substances and, in particular, reducing substances that occur in normal and pathologic serum and urine and which might interfere with the proposed tests have been investigated.

With the exception of the molybdate test, no interference occurs with

the proposed tests for bile pigments in the presence of the following substances: Glucose and lactose; ascorbic acid, thiamine hydrochloride, riboflavin, and nicotinic acid; oxalates, tartrates, and citrates; urea and uric acid; formaldehyde, acetone, and phenol; creatine and creatinine; amino-acids such as alanine and cysteine hydrochloride; chloroform, ethyl alcohol, and ether.

The molybdate test is interfered with only in the presence of glucose in excess, ascorbic acid, cysteine hydrochloride, and hydroquinone.

Detection of Bilirubin in Pathologic Serum (Bilirubinemia)

All the following tests are carried out on a white porcelain slab.

I. To 2 drops of the pathologic serum add 2 drops of the potassium ferricyanide reagent; a blue or green precipitate appears according to the bilirubin content in the sample. With normal serum a white precipitate is obtained.

II. To 2 drops of the pathologic serum add 2 drops of saturated ammonium molybdate solution and 2 drops of 60% (v/v) conc. H_2SO_4; a blue color appears. Normal serum gives a white precipitate.

III. To 2 drops of the pathologic serum add 2 drops of the diazo reagent prepared from any of the following aromatic amino compounds and mix well with a clean glass rod. A pink or purple color which may show a violet tint is immediately observed or appears after the addition of 2 drops of 2% NaOH solution as shown below. With normal serum no color develops or only a yellow color appears upon the addition of 2% NaOH.

When 2 drops of the diazo reagent are treated with 2 drops of 2% NaOH no color or a yellow color only develops.

Diazo Reagent of	*2% NaOH*	*Pathologic Serum*	*Normal Serum*
p-Nitroaniline	—	Pink	Colorless
p-Aminobenzoic acid	—	Purple-violet	Colorless
p-Toluidine	2 drops	Purple-violet	Yellow
Anthranilic acid	2 drops	Purple-violet	Pale yellow
Sulfanilamide	—	Brilliant pink	Colorless
Sulfaguanidine	—	Purple-violet	Colorless
Sulfadiazine	—	Purple-violet	Colorless
Sulfathiazole	—	Purple-violet	Colorless

In the case of the diazo reagent prepared from sulfa drugs, if 2 drops of 2% NaOH are added, a blue color is obtained with pathologic serum while a yellow color is observed with normal serum.

Comparative sensitivity of the proposed oxidation and diazo tests, to

Table 3. Comparative Sensitivity of Proposed and Some Previously Known Tests After 5 Minutes on Pathologic Serum

Tests	*Dilution*		
	1/10	1/20	1/40
Oxidation			
Fouchet's test	+	Trace	—
Nitrite test	+	Trace	—
Potassium ferricyanide test	+	+	Trace
Ammonium molybdate test	+	Trace	Trace
Diazo Reagent of:—			
Sulfanilic acid	+	Trace	—
p-Nitroaniline	+	—	—
p-Aminobenzoic acid	+	—	—
p-Toluidine	+	Trace	—
Anthranilic acid	—	—	—
Sulfanilamide	+	+	Trace
Sulfadiazine	+	+	—
Sulfaguanidine	+	+	Trace
Sulfathiazole	+	Trace	—

All tests gave positive results on dilutions of serum less than 1/10.

detect bilirubin in pathologic serum, with the previously known Fouchet, nitrite, and Ehrlich diazo tests respectively is shown in Table 3.

Detection of Bilirubin in Pathologic Urine (Obstructive Jaundice)

I. To 2 ml. of pathologic urine in a clean test tube add 2 ml. of the potassium ferricyanide reagent, and mix well by shaking; a greenish-blue color develops immediately. To 2 ml. of normal urine, add 2 ml. of the reagent and shake well; a yellow color appears.

II. To 2 ml. of pathologic urine, add 1 ml. of saturated ammonium molybdate solution and add carefully down the side of the test tube, 3 ml. of 60% (v/v) H_2SO_4. A green ring appears at the interface of the two liquids and the green color gradually spreads in the upper aqueous layer. On shaking, the whole solution assumes a dull green color, and a bluish-green precipitate deposits on standing.

With normal urine, under parallel conditions, a violet ring appears gradually after a while at the interface of the two liquids; on shaking a violet color appears in the whole solution, and on standing a violet precipitate is deposited.

III. To 2 ml. of pathologic urine, add from 3 to 12 drops of 1% (w/v) aqueous *N*-bromosuccinimide (2, 3) or *N*-chlorosuccinimide (4) solution according to the bilirubin content, drop by drop. With shaking a bluish-

green color develops and changes to olive green on standing. With normal urine, under parallel conditions, no change in the color occurs.

IV. To 2 ml. of pathologic urine, add 3 to 10 drops of the diazo reagent prepared from any of the aromatic amino compounds, drop by drop, with shaking. A purple or reddish-purple color is formed, either immediately or after the addition of 10 drops of 2% NaOH as stated below; the color does not change on standing. With normal urine, under parallel conditions, the color is unaffected or becomes deep yellow or faint orange.

A blank using water instead of urine, treated with the diazo reagent and 2% NaOH, remains colorless or assumes a yellow, pale pink, or faint orange color.

Diazo Reagent of	*No. of drops*	*2% NaOH*	*Pathologic Urine*	*Normal Urine*
p-Aminobenzoic acid	3	–	Purple	No change
p-Nitroaniline	5	–	Reddish purple	Deep yellow
m-Nitroaniline	5	–	Reddish purple	Faint orange
p-Toluidine	10	+	Reddish purple	No change
Anthranilic acid	10	+	Reddish purple	No change
α- or β-Naphthylamine	10	+	Reddish purple	Orange & turbid
Aniline	10	+	Reddish purple	Yellow & turbid
o-Toluidine	10	+	Reddish purple	Yellow & turbid
m-Toluidine hydrochloride	10	+	Reddish purple	Pale yellow and turbid
Sulfanilamide	10	–	Reddish purple	Orange
Sulfaguanidine	10	–	Reddish purple	Orange yellow
Sulfadiazine	10	–	Reddish purple	Orange yellow
Sulfathiazole	10	–	Reddish purple	Orange yellow

Comparison of the sensitivity of the proposed oxidation and diazo tests, to detect bilirubin in pathologic urine, with the previously known Fouchet, Obermeyer, iodine test, and diazotized sulfanilic acid test respectively is described in Table 4.

Application of Spot Tests for Bile Pigments on Urine

Spot tests based on oxidation or coupling are proposed to detect bilirubin in urine. Precipitation of the bile pigments is effected by adding, to 25 ml. of the urine sample, a saturated solution of barium chloride (about 5 ml.) and then filtering. Two drops of the reagent are sufficient to detect bilirubin.

Test	*Pathologic Urine*	*Normal Urine*
Potassium ferricyanide	Blue-green	–
N-Bromosuccinimide	Olive green	–
N-Chlorosuccinimide	Olive green	–
p-Nitroaniline (diazochloride)	Purple	–
p-Aminobenzoic acid (diazochloride)	Purple after 5 minutes	–

Table 4. Comparison of the Sensitivity of Proposed Tests with Some Previously Accepted Tests after 5 Minutes on Pathologic Urine (Obstructive Jaundice)

Tests	Dilution									
	1/1	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
Oxidation										
Fouchet's test	+	+	Tr.	..	..	..	..	..	..	..
Obermeyer's test	+	+	Tr.	..	..	..	..	..	..	..
Iodine test	+	+	+	Tr.	..	..	..	..	..	..
Potassium ferricyanide test	+	+	+	Tr.	..	..	..	..	..	..
Ammonium molybdate test	+	+	+	+	..	..	..	..	..	..
N-Bromosuccinimide test	+	+	Tr.	..	..	..	..	..	..	..
Diazo Reagent of:—										
Sulfanilic acid	+	+	+	+	+	+	Tr.	Tr.	..	..
p-Aminobenzoic acid	+	+	+	+	+	+	Tr.	..	..	..
p-Nitroaniline	+	+	+	+	+	+	+	Tr.	Tr.	..
p-Toluidine	+	+	+	+	+	+	Tr.	..	..	..
Anthranilic acid	+	+	+	+	+	Tr.	..	..	..	..
α- & β-Naphthylamine	+	+	Tr.	..	..	..	..	..	..	..
Aniline	+	+	+	+	Tr.	..	..	..	..	..
Sulfanilamide	+	+	+	+	+	+	+	Tr.	Tr.	..
Sulfaguanidine	+	+	+	+	+	+	Tr.	..	..	..
Sulfadiazine	+	+	+	+	+	+	+	Tr.	Tr.	..
Sulfathiazole	+	+	+	+	+	+	+	+	+	Tr.

Urine from patient with intense obstructive jaundice was diluted with mixed routine hospital urine.

When 2 drops of 7% potassium diehromate solution and 2 drops of 60% (v/v) H_2SO_4 are added to the precipitate, both the pathologic and normal samples show the same play of colors (green, blue, and purple).

DISCUSSION

For the detection of bilirubin by the proposed tests two principles are involved: (a) the controlled oxidation of bilirubin to biliverdin and (b) the formation of azobilirubin.

The controlled oxidation of bilirubin to biliverdin by ferric chloride in trichloroacetic acid (Fouchet's reagent) (5) or hydrochloric acid (Obermeyer's reagent) (6) has proved superior to the older method of progressive oxidation by nitric acid or iodine of Foord (7). Several tests have already been reported depending on the oxidation principle, but in this investigation only the following common tests have been considered, *viz.* the well-known Gmelin, Fouchet, Nakayama (8), and Obermeyer (6) tests. Comparative sensitivity of these tests and the proposed tests towards the detection of bilirubin in pure solution is shown in Table 1.

New tests based on the formation of azobilirubin by various modifications of Ehrlich's diazo reagent have been devised. Some of these surpass the most sensitive and highly specific tests available at present (see Table 2), except the diazo tablet test which requires no special equipment.

We have tried again the diazochlorides of the amines previously tested by Clarence E. May *et al.* (1), as well as those of other aromatic amines, e.g. sulfonamides, and were able to obtain stable and highly colored products. When bilirubin was dissolved in neutral alcohol, the color did not appear with the diazochlorides of any of the previously tested amines; the color appeared only after adding one drop of 2% NaOH. In a blank experiment, when 1 ml. of neutral alcohol was treated with 2 drops of the diazochloride of either, for example, *p*-toluidine or anthranilic acid, even on adding one drop of 2% NaOH, the solution remained colorless; with other diazochlorides, the solution assumed a yellow or very faint purple color.

It has already been reported that bilirubin dissolves only in alkaline solution (*p*H 8.9–14); in neutral and acid media it is precipitated. The diazo reaction therefore is positive only in alkaline solution; in acid solution no compound with the diazo reagent is formed. The reaction with bilirubin of bile or urine, on the other hand, occurs at all *p*H values (1.0–14) except in solutions highly acid with inorganic acids (9).

Comparison of the sensitivity of the proposed tests with some previously and most generally accepted tests for bile pigments on pathological serum and urine is reported in Tables 3 and 4.

SUMMARY

1. New tests proposed for the detection of bilirubin have been described.

2. Application of some of the proposed tests on pathologic and normal serum and urine have been demonstrated.

3. The diazochlorides of certain amines previously reported as failures to detect bilirubin have been shown to be successful and have been applied to bile, serum, and urine.

4. Comparative sensitivity of the proposed tests and the most generally accepted tests for bilirubin in test solutions, pathologic serum, and urine have been given.

5. Spot tests suitable for the detection of bilirubin in pathologic urine have been presented.

REFERENCES

1. May, C. E., Martindale, R., and Boyd, W. F., *J. Biol. Chem.* **104**, 255 (1934).
2. Fieser, L. F., and Rajagopalan, S., *J. Am. Chem. Soc.* **71**, 3935 (1949).
3. Barakat, M. Z., and Mousa, G. M., *J. Pharm. and Pharmacol.* **4**, 115 (1952).
4. Hebbelynck, M. F., and Martin, R. H., *Experientia* **5**, 69 (1949).
5. Fouchet, A., *Compt. rend. soc. biol.* **80**, 826 (1917); *J. pharm. chim.* **18**, 19 (1918).
6. Kapsinow, R., *J. Am. Med. Assoc.* **82**, 687 (1924); *Chem. Abstr.* **18**, 1682 (1924).
7. Foord, A. G., and Baisinger, C. F., *Am. J. Clin. Pathol.* **10**, 238 (1940).
8. Mathews, A. P., *Physiologic Chemistry* (ed. 4), New York, Wood, 1925, p. 1155.
9. Shelagurov, A. A., *Chem. Zentr.* **1**, 2194 (1937); *Chem. Abstr.* **32**, 9133 (1938).

Urobilinogen Excretion in Normal Adults

Results of Assays with Notes on Methodology

Bernard Balikov

THE QUANTITATIVE ESTIMATION OF UROBILINOGEN in feces and urine in the evaluation of hepatic and hemolytic diseases has become widely accepted as a useful tool both for clinical and research purposes. Our interest in urobilinogen assays stemmed principally from a study of hepatic function subsequent to major thermal trauma. Of the several methods available for this analysis, the one based on the works of Watson and his associates is probably most widely used, and was the one employed in this study. However, several significant changes in methodology have taken place since Watson first published his normal ranges in 1937 (1). Consequently, it was felt that a reassessment of these values would improve the reliability of any interpretations we made of our findings.

This report principally concerns the results of assays on 46 adults for urine urobilinogen and 42 for fecal urobilinogen utilized so as to reveal statistically valid information concerning normal values for this modified procedure.

MATERIAL AND METHODS

Subjects were assumed to be normal if they had no illness at the time samples were collected, and had no history of jaundice, gastrointestinal ulcers, malaria, or blood dyscrasias. Samples were not collected on

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females during menstrual periods. Subjects were between 18 and 61 years of age and were divided into four groups, which will hereafter be referred to by their group letters: Group A, males weighing 70 kg. and less; Group B, males weighing more than 70 kg.; Group C, females weighing less than 56 kg.; and Group D, females weighing 56 kg. and more.

Urobilinogen in both urine and feces was assayed by the quantitative methods of Schwartz, Sborov, and Watson (2) as modified by Balikov (3). The standard curve using pontacyl dyes was used as described by Watson and Hawkinson (4), and readings were made at 565 mμ on the Coleman Junior spectrophotometer, Model 6A, using 19 x 150 mm. cuvets. It should be noted that these methods involve reduction with $Fe(OH)_2$ and subsequent extraction with petroleum benzin as differentiated from the "rapid" methods which do not include either or both of these steps.

Urine assays were all made on aliquots of 24-hour collections. Samples were collected in painted or paper-covered jugs which contained 5 Gm. of anhydrous Na_2CO_3 and 100 ml. of petroleum benzin. All analyses were completed the day the collection was finished.

Feces were collected over a 4-to-5-day period in weighed, waxed containers with airtight lids. Samples were stored in a refrigerator until the collection was complete. Aliquots were taken from a blend made by mixing all samples collected during a single period in a Waring Blendor with measured amounts of water sufficient for easy stirring. Assays were usually completed the day the last sample of a series was collected. In no case were analyses postponed longer than one more day.

Notes on the Method

The literature frequently refers to the use of "saturated sodium acetate" without specifying whether to use the anhydrous or triple-hydrated salt. Since the function of the acetate is to convert the free HCl to acetic acid (5) it was felt that perhaps either may be used. To check this possibility, saturated solutions were made of both salts by simply adding an excess to water, so that some undissolved solid was always present. The two acetate solutions were used on identical aliquots of the same ferrous hydroxide filtrates. Two urine and 4 fecal samples showed no determinate difference between the two solutions (Table 1). Thus, the less expensive triple-hydrated salt prepared as indicated above was used exclusively in this work.

It has been reported that the urobilinogen-aldehyde color fades on standing and that the stability of this color depends to some extent on the purity and amount of sodium acetate used (2). Using only reagent

TABLE 1. A COMPARISON OF ANHYDROUS VS. TRIPLE-HYDRATED SODIUM ACETATE

Sample	Per cent transmittance	
	Anhydrous sodium acetate	*Triple-hydrated sodium acetate*
Urine #1	100.0	99.1
Urine #2	92.2	94.7
Feces #1	74.1	71.2
Feces #2	59.2	60.1
Feces #3	85.8	85.1
Feces #4	75.1	74.3

TABLE 2. STABILITY OF THE FINAL UROBILINOGEN-ALDEHYDE COLOR

Time after formation of color (min).	*Per cent transmittance*[a]					
	Sample 1		*Sample 2*		*Sample 3*	
	Light	*Dark*	*Light*	*Dark*	*Light*	*Dark*
6	..	..	79.2	78.2	57.0	57.0
7	90.0	90.6	..	..	..	..
10	90.2	90.3	79.7	79.0	57.0	57.0
15	90.1	89.7	79.2	79.0	57.0	57.0
20	89.9	90.1	79.2	79.0	57.0	57.0
25	90.0	89.9	79.0	78.9	57.0	57.0
30	89.8	90.0	79.1	79.0	57.0	57.0
70	..	..	..	..	57.0	57.0
75	90.6	90.2	79.2	79.8[b]	..	..

[a] Readings were made with 19 x 150 mm. cuvets.

[b] This sample was not in the dark during the period 30 to 75 minutes.

grade triple-hydrated sodium acetate in a 3:1 ratio to the Ehrlich's reagent, this color was found to be stable for at least 70 minutes both in the dark and in ordinary artificial light (Table 2).

A check was made for the completeness of the extraction of urobilinogen with petroleum benzin from the acidified $Fe(OH)_2$ filtrate. After several trials in which the number and time of extractions were varied it was found that using 35 ml. of petroleum benzin, a single, vigorous extraction for 7 minutes would completely extract the urobilinogen. A second extraction made on several normal and pathologic urine and fecal specimens consistently showed 99 per cent transmittance or more. However, it is strongly recommended that the individual actually performing this assay make a similar check for completeness of extraction.

It has been found to be of utmost importance to complete an extraction with the modified Ehrlich's reagent before the sodium acetate is added. If the two reagents are added simultaneously, results will be from 50 to 75 per cent lower.

RESULTS

Urine

Using the t test for significance (6), Groups A, B, C, and D were compared with each other in an attempt to determine if there were any significant differences between their means (Table 3). Since all P values are greater than 0.05, all four groups may be considered parts of the same population. However, the mean of Group A (1.2) is twice as high as that of the next highest group (Group B = 0.6), which indicates an unexplained tendency for higher values in lighter-weight males.

TABLE 3. SIGNIFICANCE OF THE DIFFERENCES OF THE MEANS OF URINE UROBILINOGEN EXCRETION BETWEEN VARIOUS POPULATION GROUPS

	A vs. B	*A vs. C*	*A vs. D*	*B vs. C*	*B vs. D*	*C vs. D*
Mean	1.2 | 0.6	1.2 | 0.4	1.2 | 0.5	0.6 | 0.4	0.6 | 0.5	0.4 | 0.5
No. of samples	14 | 10	14 | 11	14 | 11	10 | 11	10 | 11	11 | 11
Combined SD	1.06	0.93	0.97	0.34	0.45	0.49
t	1.27	1.96	1.65	0.79	0.30	0.33
P[a]	0.2–0.3	0.1–0.05	0.1–0.2	0.4–0.5	0.7–0.8	0.7–0.8

[a] From Fisher, R. A., *Statistical Methods for Research Workers*, New York, Hafner Pub. Co., 1948, p. 174.

The 46 urine urobilinogen values were checked for normalcy of distribution by a plot against the Normal Equivalent Deviate values according to the method of Moore and associates (7). By inspection, the distribution did not appear normal, but a plot of the logarithms of these urobilinogen values does so appear (Fig. 1). Thus, these logarithm values were used in calculating the normal range. The pertinent results are as follows:

$$\text{Mean} = 0.45$$

$$\text{Standard deviation (SD)} = \pm 0.425$$

$$\text{Normal range (mean} \pm 2\text{x (SD)} = -1.30 \text{ to } 0.40$$

Conversion of these logarithms to their antilogarithms yields the following results:

$$\text{Mean (more correctly, Median)} = 0.36 \text{ mg./24 hr.}$$

$$\text{Normal range} = 0.05 \text{ to } 2.5 \text{ mg./24 hr.}$$

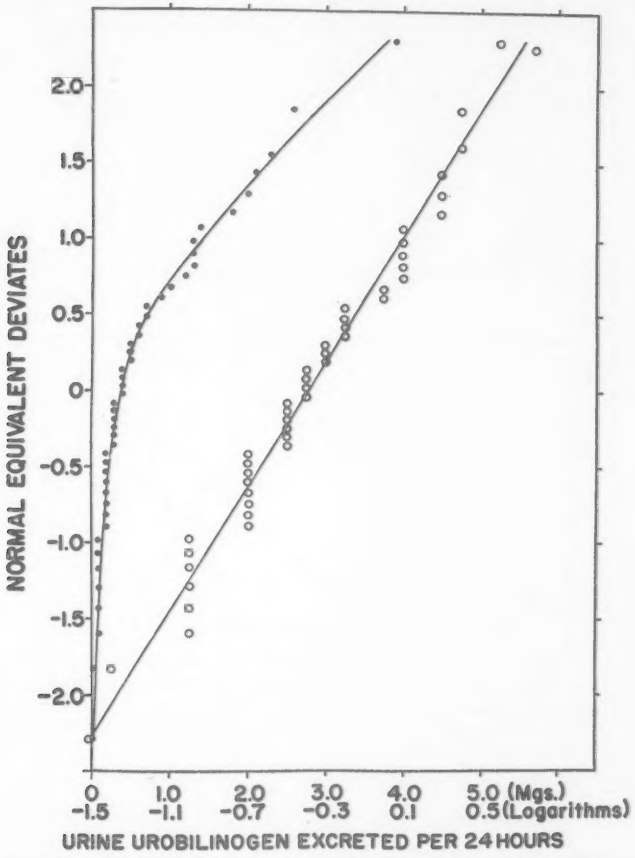

Fig. 1. Lognormal distribution of urine urobilinogen values on adults. The dots represent values in terms of milligrams/24 hours, the circles represent the logarithms of these values. The lines are drawn to emphasize the curvature of the one set of points compared with the relative linearity of the other.

Feces

As for urine, the *t* test for significance was used to compare the fecal excretion of urobilinogen of the four groups (Table 4). Although differences significant at the 1% level are found between three of the pairs (A vs. C, B vs. C, and B vs. D), no significant differences are found between the male groups (A and B) or the female groups (C and D). There-

TABLE 4. SIGNIFICANCE OF THE DIFFERENCES OF THE MEANS OF FECAL UROBILINOGEN EXCRETION BETWEEN VARIOUS POPULATION GROUPS

	A vs. B	*A vs. C*	*A vs. D*	*B vs. C*	*B vs. D*	*C vs. D*
Mean	103 | 122	103 | 37	103 | 70	122 | 37	122 | 70	37 | 70
No. of samples	10 | 11	10 | 11	10 | 10	11 | 11	11 | 10	11 | 10
Combined SD	34.6	28.9	45.5	26.5	43.0	38.1
t	1.26	5.23	1.62	7.54	2.77	1.98
P[a]	0.2–0.3	0.01	0.1–0.2	0.01	0.01–0.02	0.05–0.10

[a] From Fisher, R. A., *Statistical Methods for Research Workers*, New York, Hafner Pub. Co., 1948, p. 174.

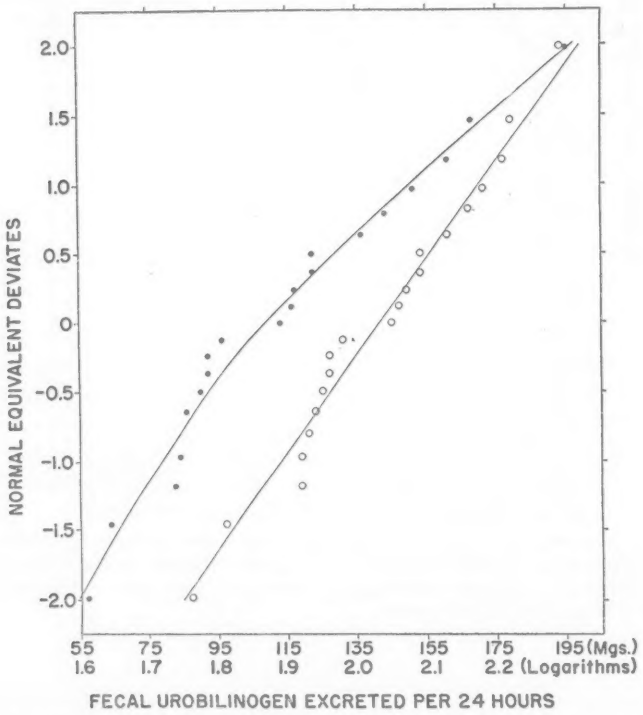

Fig. 2. Lognormal distribution of fecal urobilinogen values on adult males. The dots represent values in terms of milligrams/24 hours, the circles represent the logarithms of these values. The lines are drawn to emphasize the curvature of the one set of points compared with the relative linearity of the other.

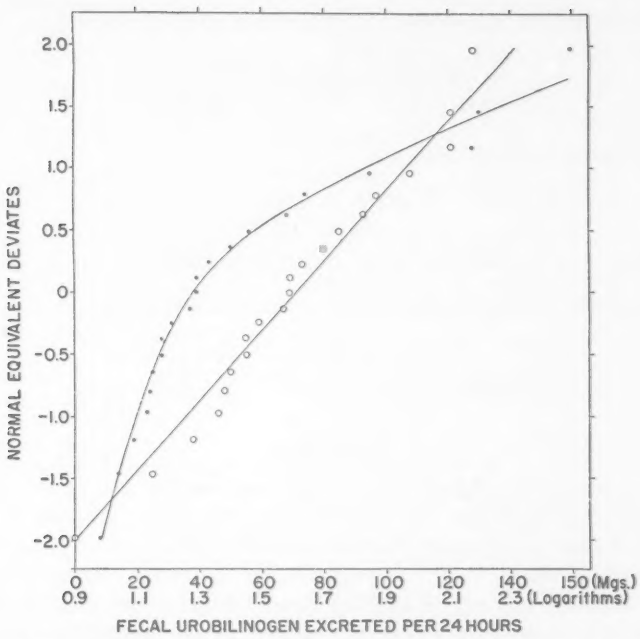

Fig. 3. Lognormal distribution of urine urobilinogen values on adult females. The dots represent values in terms of milligrams/24 hours, the circles represent the logarithms of these values. The lines are drawn to emphasize the curvature of the one set of points compared with the relative linearity of the other.

fore, all males were combined and compared with all females. These groups were found to be different at a level greater than 99 per cent ($P < .01$).

The normalcy of distribution of the values for each of the two groups (adult males and adult females) was checked as for urine urobilinogen above. It is apparent from Figs. 2 and 3 that the logarithms of the values for both groups are more normally distributed than the values themselves. As for urines, then, the logarithms were used in calculating the normal ranges. The pertinent results for males are:

$$\begin{aligned} \text{Mean} &= 2.032 \\ \text{SD} &= \pm 0.138 \\ \text{Normal range} &= 1.756 \text{ to } 2.308 \end{aligned}$$

Conversion of the logarithms to their antilogarithms yields:

Median = 101 mg./24 hr.
Normal range = 57 to 200 mg./24 hr.

The results on adult females are:

Mean = 1.608
SD = ±0.328
Normal range = 0.952 to 2.264

Conversion of the logarithms to their antilogarithms yields:

Median = 40 mg./24 hrs.
Normal range = 8 to 150 mg./24 hr.

DISCUSSION

The grouping of subjects by sex, age, and weight was based on the following considerations. The urobilinogen found in urine and feces is formed at least in large part from bile which in turn is formed from hemoglobin (8). Since the blood hemoglobin concentration varies with age (up to 16 years) and sex (9) and the blood volume varies with weight, it was felt that these factors may be variables in the production and consequent elimination of urobilinogen. Our subjects were all over 18 and the values reported here should not be applied to children without further investigation. The mean weights of 70 kg. for men and 56 kg. for women were taken from data reported in Sunderman and Boerner (10).

There is no apparent explanation for the logarithmic distribution of normal values. This type of distribution has been noted for a number of blood constituents including bilirubin (11) and so perhaps should not be surprising when found in excretory pigments as well.

Since it has been shown that apparent minor changes in method can result in significant differences in results (3), the normal values reported here should not be applied to results obtained using any but the described procedure, unless further investigation proves such an application to be valid.

SUMMARY

Using the quantitative methods of Schwartz, Sborov, and Watson, as modified by Balikov, normal ranges for excretion of urobilinogen have been determined for adults. The individual values fall into a logarithmic type of distribution.

The normal range for urine urobilinogen excretion is from 0.05 to 2.5 mg./24 hours, with a median of 0.36. The excretion of fecal urobilinogen ranges from 57 to 200 mg./24 hours for males and from 8 to 150 mg. for females. The medians for these groups are 101 and 40 mg., respectively.

REFERENCES

1. Watson, C. J., *Arch. Internal Med.* **59**, 196 (1937).
2. Schwartz, S., Sborov, V., and Watson, C. J., *Am. J. Clin. Path.* **14**, 598 (1944).
3. Balikov, B., *Clin. Chem.* **1**, 264 (1955).
4. Watson, C. J., and Hawkinson, V., *Am. J. Clin. Path.* **17**, 108 (1947).
5. Terwen, A. J. L., referred to by Watson, C. J., *Arch. Internal Med.* **47**, 698 (1931).
6. Mainland, D., *Elementary Medical Statistics*, Philadelphia, Saunders, 1952, p. 156.
7. Moore, F. V., Cramer, F. B., and Knowles, R. G., *Statistics for Medical Students*, Philadelphia, Blakiston, 1951, pp. 19–21.
8. Cantarow, A., and Trumper, M., *Clinical Biochemistry* (ed. 4), Philadelphia, Saunders, 1949, p. 421.
9. Sunderman, F. W., and Boerner, F., *Normal Values in Clinical Medicine*, Philadelphia, Saunders, 1950, pp. 37–41.
10. *Idem*, pp. 633–634.
11. Wootton, I. D. P., and King, E. J., *Lancet* **1**, 470 (1953).

Paper Chromatography of Free Amino Acids in Human Blood Serum

Vladimír Jirgl

BOULANGER and BISERTE (1) used a solution of acid acetone for the extraction of amino acids in biologic material. The separation of amino acids on buffered papers described by *Rybář*, *Toušek*, and *Hais*, (2) and *Hais* and *Horešovský* (3) used the combination of tertiary butanol-borate buffer system (*p*H 8.5) for separation of amino acids on paper.

In our laboratories we have developed a chromatographic method for the separation of free amino acids in human blood serum in which previous desalting is not necessary.

One milliliter of blood serum is deproteinized with 9 ml. of ethanol-acetone (7:3 [V/V]). After centrifugation the whole supernatant is

Table 1. R_f VALUES OF TYPICAL CHROMATOGRAM

Amino acid	
Peptide?	0.07
l-Cystine	0.085
Histidine	0.13
Glycine	0.28
Glutamic acid	0.33
d-l-Alanine	0.60
Proline	0.62
Tyrosine	0.64
Tryptophane	0.71
Valine	0.78
d-l-Phenylalanine	0.81
i-Leucine	0.86

Solvent front, 42 cm.; time, 8 hr.; temperature, 20°.

From the Central Biochemical Laboratories of University Hospital, Prague, Czechoslovakia.

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Fig. 1. Paper, Whatman No. 3; mobile phase, n-butanol-methanol-formic acid 6:1:3 (V/V/V/); stationary phase, water; chromatogram development, 0.1% butanolic solution of ninhydrin.

evaporated and the residue extracted 3 times with 2-ml. portions of 96 per cent ethanol, and the combined extracts are evaporated again.

The residue of the ethanol extraction is dissolved in 0.06 ml. of hot 70 per cent ethanol. After centrifugation an aliquot part (0.03 ml. equivalent to 0.5 ml. of blood serum) was spotted on Whatman No. 3 paper, length 50 cm., in lines 1 cm. wide. The chromatographic chamber (20 x 30 x 50 cm.) is saturated with about 100 ml. water. The separation is made at 20°, 7–8 hours. The mobile phase used is: *n*-butanol-methanol-formic acid 6:1:3 (V/V/V/). After drying the paper strips 1 hour at 70–80° the chromatogram is developed with 0.1 per cent solution of ninhydrin in *n*-butanol at 70–80°.

Results are shown in Table 1 and Fig. 1.

SUMMARY

A method has been described for the chromatographic separation on paper of free amino acids in human blood serum in which the previous desalting is not necessary.

REFERENCES

1. Boulanger, P., and Biserte, G., *Bull. Soc. Chim. Biol.* **31,** 696 (1949).
2. Rybář, D., Toušek, B., and Hais, I. M., *Chemické listy* **10,** 1532 (1954).
3. Hais, I. M., and Horešovský, O., *Chemické listy* **4,** 549 (1954).

Comparison of Plasma Uric Acid Levels Obtained with Five Different Methods

Eugene L. Kanabrocki, Joseph Greco, Lee Wilkoff, and Roger Veach

THE PURPOSE OF THIS STUDY was to compare the plasma uric acid levels obtained by five accepted methods, and also to select a method most favorable for routine analysis. As the basis for such a selection, the following criteria have been considered: favorable comparison with the uricase method, ease of performance, and stability of the reagents. The selected method has been used to establish a normal uric acid level and also to study the variation of uric acid levels in normal subjects over an extended period of time.

EXPERIMENTAL METHODS, MATERIAL, AND RESULTS

Plasma was collected from 12 fasting, apparently normal subjects and from 2 patients with osteoporosis. Plasma was used in all the studies because the interfering materials appear to be present largely in the red cells of the blood, because the present methods are not well adapted to the analysis of the cells, and because the concentration of uric acid in plasma is about twice as great as the concentration in cells (1). Immediately and simultaneously the determinations of uric acid were begun, using the methods of Brown (2), Newton (3), Kern and Stransky (4), Folin (5), and Blauch and Koch (6).

Normal Plasma Uric Acid Level

Using the method of Kern and Stransky (4) the uric acid determinations were made on plasma of 22 additional subjects, 14 normal and 8 without any history of nephritis or collagen disease. The average normal plasma uric acid concentration of these 22 subjects and 12 normal subjects used in the study presented in Table 1, is 4.05 mg. per 100 ml. with

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Table 1. Plasma Uric Acid Levels in mg. per 100 ml. Obtained by Five Different Methods

Subject	Sex	Age	Method				
			Brown	Newton	Kern and Stransky	Folin	Blauch and Koch
1	F	56	5.40	2.68	3.10	2.80	2.04
2	F	29	6.80	4.86	4.30	4.90	3.90
3	F	29	2.60	3.86	3.50	2.80	1.90
4	F	23	6.28	3.52	3.35	3.84	2.86
5	M	63	5.00	4.24	3.46	4.20	3.30
6	M	36	8.38	5.44	4.36	6.00	5.16
7	M	31	3.35	2.60	2.70	2.90	2.26
8	M	30	6.05	4.86	3.95	6.02	4.78
9	M	30	6.92	5.24	4.36	5.80	5.30
10	M	29	9.20	6.00	4.30	6.00	5.16
11	M	27	6.55	5.40	4.90	6.92	5.18
12	M	26	5.55	3.66	3.75	4.34	3.58
13	M	48	6.55	5.54	4.38	6.34	4.68
14	M	55	3.75	2.98	2.80	3.20	2.44
Average			5.88	4.34	3.80	4.71	3.75
Standard Deviation			2.00	1.16	0.67	1.35	1.17

Table 2. Plasma Uric Acid Levels in mg. per 100 ml. in Normal Subjects Over a Period of 7 Weeks

Subject	Weeks				
	1	3	5	6	7
1	5.08	5.35	5.75	5.92	5.20
2	4.05	3.15	4.67	4.15	4.27
3	4.75	5.31	5.16	5.30	5.05
4	3.85	3.77	4.53	3.70	3.55
5	3.26	3.25	3.80	4.08	3.35

a normal range of 2.7–5.9 mg. per 100 ml. Plasma from 3 patients suffering from gout and under treatment, gave 9.86, 6.10, and 8.84 mg. per 100 ml.

Variations in Uric Acid Levels Over a Period of Time

Plasma was collected from 5 normal fasting male subjects at intervals of 1, 3, 5, 6, and 7 weeks (Table 2). Uric acid determinations were carried out employing the selected method (4).

DISCUSSION

Although the uricase method can be considered as most specific for the determination of plasma uric acid, it has a disadvantage in that the final stage is based on a nonspecific colorimetric reaction. The method of

Blauch and Koch (6) is a uricase method and is based upon colorimetric analysis of plasma before and after it has been subjected to the action of the specific enzyme uricase. The other four methods (2, 3, 4, 5) are based on direct colorimetric reaction with the plasma filtrate.

Examination of the data listed in Table 1 appears to favor the methods of Newton (3) and Kern and Stransky (4) in that their values are considerably closer to the values obtained by the method of Blauch and Koch (6). Evaluation of these two methods with regard to details of procedure indicated that the method of Kern and Stransky (4) would be more desirable, specifically because its reaction time is less restricted and the reagents are stable and nonpoisonous. The normal uric acid level of 4.0 mg. per 100 ml., with a range of 2.7–5.9 mg. per 100 ml. obtained with this method, compares very favorably with the normal values reported in the literature (7, 8).

The differences between the highest and the lowest uric acid levels for the period of 7 weeks were 0.84, 1.52, 0.56, 0.98, and 0.83 mg. per 100 ml., respectively, for the 5 subjects under study. There does not appear to be any definite trend in uric acid levels, in any of the subjects under study (see Table 2). The variations that do occur might be due to variations in diet or physical activity. McEwen (10) has shown that the diets high in purines or fat raise the blood uric acid. Rakestraw (11) found that severe exercise is followed by a rise of uric acid as much as 0.9 mg. per 100 ml. The physical activities and the dietary habits were not rigorously controlled in the study presented here; nevertheless, they were the usual activities and habits of the subjects. It is known that certain drugs, particularly the salicylates (12), reduce the concentration of blood uric acid. None of the subjects took salicylates or any other drugs during the studies.

A similar comparison study using three different procedures was reported by Lous and Sylvest (9). The methods involved in this study were the colorimetric method of Folin, the titrimetric method of Brøchner-Mortensen, and the uricase method of Praetorious. They found that no correlation between the results could be demonstrated. The colorimetric procedure of Folin gave lower results while the titrimetric procedure of Brøchner-Mortensen gave results higher than those obtained with the uricase method of Praetorious.

SUMMARY

1. A comparison has been made of plasma values obtained by 5 different methods for uric acid estimation.

2. According to the uricase method, the plasma concentration of uric

acid in fasting normal subjects ranged from 1.9–5.1 with average of 3.7 mg. per 100 ml.

3. The closest agreement with the uricase method was obtained with the method of Kern and Stransky which gave normal values ranging from 2.8 to 4.9 with an average of 3.8 mg. per 100 ml.

4. A variation of about 25 per cent was observed in the plasma uric acid levels over a period of 7 weeks.

REFERENCES

1. Wu, H., *J. Biol. Chem.* **51**, 21 (1921).
2. Brown, H., *J. Biol. Chem.* **158**, 601 (1945).
3. Newton, E. B., *J. Biol. Chem.* **120**, 315 (1937).
4. Kern, A., and Stransky, E., *Biochem. Ztschr.* **290**, 419 (1937). Modified by Archibald; *Department of the Army Technical Manual* TM 8—227, 1951, p. 232.
5. Folin, O., *J. Biol. Chem.* **54**, 153 (1922).
6. Blauch, M. B., and Koch, F. S., *J. Biol. Chem.* **130**, 443 (1939).
7. Jacobson, B. M., *Ann. Internal Med.* **11**, 1277 (1938).
8. Bulger, H. A., and Johns, H. E., *J. Biol. Chem.* **140**, 427 (1941).
9. Lous, P., and Sylvest, O., *Scand. J. Clin. & Lab. Invest.* **6**, 40 (1954).
10. McEwen, C., *J. Mt. Sinai Hosp. N. Y.* **854** (1942).
11. Rakestraw, N. W., *J. Biol. Chem.* **47**, 565 (1921).
12. Fine, M. S., and Chase, A. F., *J. Biol. Chem.* **21**, 371 (1915).

Interference of Bromide in the Zak Ferric Chloride-Sulfuric Acid Cholesterol Method, and Means of Eliminating This Interference

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THE AUTHORS' LABORATORY has employed the modified Zlatkis method (1) of Zak and associates (2) for the routine determination of serum cholesterol fractions for over a year with generally satisfactory results. However, ester fractions greater than 80 per cent of the total cholesterol values were occasionally obtained. Recently, an analysis of the serum "total cholesterol" from a patient with acute brominism (serum bromide, 50 mEq./L.) resulted in the formation of a reddish-orange instead of the typical purple color. The "free cholesterol" fraction of this serum had the usual appearance.

Subsequent investigation showed that the ferric chloride-sulfuric acid color reaction for cholesterol gives erroneously high results in the presence of even traces of bromide. This interference is a likely explanation for the high ester fractions previously mentioned. Table 1 shows the effect of various quantities of NaBr added to a cholesterol standard. The interference is measurable with NaBr concentrations as low as 5 μg. or less per tube—levels at which the color appears a normal purple to the eye. In Table 2 are results of cholesterol determinations with varying amounts of NaBr added to human sera. The values for total cholesterol are incorrect and the ester fraction (calculated by subtracting the free cholesterol from the total) is in similar positive error. The free cholesterol levels are essentially unaffected because the cholesterol digitonide precipitate is freed of bromide contamination by decanting and washing with acetone. Iodide also gives a color comparable to that of bromide. Several experiments showed that neither bromide nor iodide affects the

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Table 1. Effect of Added Bromide on a Standard Solution of Cholesterol (0.08 mg./3 ml.) (Zak Method)

NaBr Added (μg./tube)	*Absorbance*[a]	*Apparent cholesterol* (mg./100 ml. serum)
None	0.370	200
6	0.430	233
15	0.440	238
30	0.485	262
60	0.515	278
120	0.580	314
240	0.615	333
480	0.670	362

[a] Beckman B Spectrophotometer, 560 mμ.

Table 2. Interference of Bromide on Zak Cholesterol Method

Serum	*NaBr added* (mEq./L.)	*Cholesterol found*			
		Total[a]	*Free*[a]	*Ester*[a]	*% of total*[b]
A	..	215	50	165	(77)
	2.0	255	55	200	(78)
B	..	257	56	201	(78)
	5.0	325	58	267	(82)
C	..	330	71	259	(79)
	12.5	403	71	332	(83)
D	..	261	64	197	(76)
	12.5	376	67	309	(82)
E	..	255	64	191	(75)
	25.0	394	66	328	(83)
F	..	256	61	195	(76)
	50.0	395	63	332	(84)
G	..	215	41	174	(81)
	100.0	325	45	280	(86)

[a] Values are given as mg. per 100 ml. serum.

[b] $\frac{\text{ester}}{\text{total}} \times 100$.

Table 3. Elimination of Bromide Interference on Zak Cholesterol Method with $AgIO_3$

Serum	*NaBr Added* (mEq./L.)	*Cholesterol found*[a]			
		No treatment		*$AgIO_3$ Treatment*	
		Total	*Free*	*Total*	*Free*
A	..	175	50	177	50
	5.0	218	51	176	49
B	..	223	60	218	58
	12.5	289	60	227	59
C	..	199	56	202	58
	25.0	295	55	200	57
D	..	219	64	207	62
	50.0	327	65	216	65

[a] Values are given as mg. per 100 ml. serum.

determination of cholesterol by the Lieberman-Burchard reaction. A detailed study of these observations was not made.

Various means for eliminating the bromide interference were attempted. Mixing the serum with a small quantity of solid silver sulfate prior to extraction with ethanol-acetone mixture is effective, although the serum may be cloudy after this treatment due to denaturation of the proteins. Cholesterol values of silver sulfate-treated bromide-containing sera averaged 95 per cent of those found before the addition of NaBr. Further studies revealed silver iodate to be even more satisfactory than silver sulfate. This is in spite of the fact that the Zak color reaction is inhibited by soluble iodates (and bromates) such as the sodium and potassium salts. The sample is shaken for about 5 minutes with approximately 25 to 50 mg. per ml. of solid silver iodate and centrifuged briefly. The clear supernatant serum is analyzed according to the Zak procedure (2). Table 3 demonstrates the elimination of the interference of bromide with silver iodate and shows that the procedure is adequate not only with low therapeutic concentrations of bromide but with toxic levels as well.

The results of the present study are significant in view of the widespread abuse of numerous patent medicines which contain bromide and the fact that bromide is very slowly eliminated from the body. It is therefore recommended that silver iodate be incorporated routinely into the Zak procedure whenever there is a possibility of bromide being present in the serum.

SUMMARY

It is shown that the ferric chloride-sulfuric acid color reaction for cholesterol gives erroneously high results in the presence of even traces of bromide. This bromide interference is eliminated by shaking the serum with silver iodate prior to extraction with ethanol-acetone.

REFERENCES

1. Zlatkis, A., Zak, B., and Boyle, A. J., *J. Lab. Clin. Med.* **41**, 486 (1953).
2. Zak, B., Dickenman, R. C., White, E. G., Burnett, H., and Cherney, P. J., *Am. J. Clin. Path.* **24**, 1307 (1954).

Simple Specific Test for Urine Glucose

Alfred H. Free, Ernest C. Adams, Mary Lou Kercher, Helen M. Free, and Marion H. Cook

PROCEDURES FOR THE DETECTION OF SUGAR IN URINE are among the oldest tests known in clinical chemistry. Legends going back to earliest times describe the attraction of insects to urine containing sugar. In many instances it is difficult to separate legend from reality in tracing the history of tests for glycosuria.

Among such pioneer American clinical chemists as Stanley Benedict, Otto Folin, Victor Myers, and Donald Van Slyke, there was great interest in establishing and using tests for the detection of sugar in the urine. From the many tests described during the early part of the twentieth century, Benedict's copper reduction test emerged as the one of greatest popularity. During the past 10 years Clinitest,[1] which is a self-heating alkaline copper reduction test, has received wide acceptance in this country and in many other parts of the world. This test will subsequently be referred to as the tablet copper reduction test.

This report describes a new, simple test for the identification of glucose in the urine. The test depends on the action of an enzyme and thus bypasses the classical principle of alkaline reduction of metallic ions. The test is called Clinistix,[2] and will hereafter be referred to as the glucose oxidase test.

METHODS

The reagents involved in the glucose oxidase test are glucose oxidase, peroxidase, and orthotolidine. They are impregnated into a strip of stiff paper. In order to carry out a test, the strip is merely dipped into the

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[1] Clinitest is a registered trademark of the Ames Company Inc., Elkhart, Ind.

[2] Clinistix is a registered trademark of the Ames Company Inc., Elkhart, Ind.

urine specimen. One minute later the strip is observed for the appearance of a blue color which indicates the presence of glucose.

The enzyme glucose oxidase was first identified by Muller (1) approximately 30 years ago. In the presence of oxygen the enzyme catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide. In the absence of oxygen the reaction of glucose and glucose oxidase may utilize certain organic compounds as hydrogen acceptors. In the glucose oxidase test the hydrogen peroxide formed reacts with orthotolidine, the reaction being catalyzed by a second enzyme—peroxidase. This reaction results in the development of a deep blue color.

EXPERIMENTAL

The glucose oxidase test is very sensitive for the detection of glucose. Less than 0.01% glucose in water will give a positive reaction. Somewhat greater amounts of glucose in urine are required for a positive reaction, with 0.01% usually being negative and 0.1% glucose in urine giving a positive reaction. As with most other urine qualitative tests, the precise sensitivity depends somewhat on the nature of the sample. Table 1 shows typical quantities of glucose which need to be added to urine to get positive reactions with the tablet copper reduction test, Benedict's qualitative test, and the glucose oxidase test. The sensitivity of the enzyme test is such that urines with a small quantity of glucose either added or naturally occurring may give a positive reaction with the glucose oxidase test, a negative reaction with Benedict's and a negative reaction with the tablet copper reduction test. Whether the great sensitivity of the new enzyme test will be an advantage or a disadvantage has not been established. Many laboratories including our own (2) have established that the high sensitivity of Benedict's test is a disadvantage over a less sensitive test. However, Benedict's test is a test for total reducing substances rather than for glucose.

Glucose oxidase has been studied for the specificity of its reaction with glucose. Keilin and Hartree (3) reported that the enzyme had a high specificity for glucose but would react slowly with mannose, xylose,

Table 1. Typical Concentrations of Glucose in Urine Required for Positive Reaction

Test	*Glucose concentration (%)*
Tablet copper reduction test	0.15
Benedict's copper reduction test	0.08
Glucose oxidase test	0.05

maltose, and galactose. The extreme sensitivity of the glucose oxidase test raises the question of whether it will react with concentrations of other sugars which may be encountered in the urine. It has been found that many commercial samples of sugars other than glucose give positive tests with the glucose oxidase test when tested in concentrated aqueous solutions. Purification of such sugars by fermentation with baker's yeast of any glucose present or by enzymatic removal of the glucose results in a nonreactive material. The sugars which have been established to be nonreactive with the glucose oxidase test at concentrations of 20% in urine are galactose, fructose, lactose, mannose, maltose, sucrose, xylose, D-ribose, D-arabinose, L-arabinose, and L-xylulose.

Glucose oxidase catalyzes the oxidation of β-D-glucose but has no effect on the oxidation of α-D-glucose. A freshly prepared solution of crystalline glucose contains only the alpha form and is essentially nonreactive with the glucose oxidase test. However, in solution an equilibrium mixture between the alpha and beta forms is readily established and within a few minutes reactivity with the glucose oxidase test appears, demonstrating the presence of β-D-glucose. In blood and urine containing glucose the equilibrium mixture of alpha and beta glucose is present so there is no problem of delayed reactivity, since β-D-glucose is available for immediate reaction with the enzyme.

In the treatment of diabetes, some clinicians regulate insulin dosage on the basis of the amount of sugar in the urine. For this reason quantitation of the amount of sugar in the urine is important. There are several factors which influence the amount of color in an enzyme test such as the glucose oxidase test. In addition to the effect produced by the sugar concentration the color is influenced by *p*H, temperature, and the concentration of antagonistic substances such as ascorbic acid. Accordingly, it has not been possible to quantitate the amount of glucose in urine. It is quite easy to demonstrate that with different amounts of sugar added to a given urine different colors are obtained. However, the variability of the urine and the inability of average operators to recognize small color differences makes it impossible to do little more than differentiate between small and large amounts of glucose in urine.

RESULTS

Table 2 shows the number of positive tests with the glucose oxidase test, the tablet copper reduction test, and Benedict's qualitative test obtained on a series of random urines from 352 healthy subjects. It will be seen that many positive reactions (trace) were obtained with Benedict's

Table 2. URINE SUGAR TESTS ON HEALTHY SUBJECTS

Test	*Positive*	*Negative*
Glucose oxidase test	2	350
Tablet copper reduction test	3	349
Benedict's qualitative	63	289

test but only 2 positive reactions were obtained with the glucose oxidase test and 3 positive reactions with the tablet copper reduction test. A series of tests were applied to the urines giving negative reactions with the glucose oxidase test and the tablet copper reduction test but trace Benedict's tests. These included paper chromatography, measurement of the amount of reducing substance in the urine with the Nelson-Somogyi procedure (4) before and after fermentation with baker's yeast, measurement of the amount of reducing substances with Nelson-Somogyi procedure in the urine before and after aeration with glucose oxidase, and attempts to form osazone crystals. The results of these tests indicated that the urines from healthy subjects which gave trace reactions with Benedict's test but negative reactions with the glucose oxidase test contained nonglucose reducing substances. Confidence in the results of such tests is achieved by reason of the fact that addition of glucose to negative urines in quantities of 0.1 per cent can readily be recognized by paper chromatography, by decrease in total Nelson-Somogyi reducing substances after yeast fermentation or after aeration with glucose oxidase, and by a positive glucose oxidase test reaction.

With some urines this quantity of glucose can be identified by forming osazone crystals, but with other urines osazone crystals have not been obtained with this small amount of glucose.

A large number of tests have been carried out over a period of more than a year on random hospital urine specimens using the glucose oxidase test and one or two of the standard copper reduction methods, the tablet copper reduction test, and Benedict's qualitative test. These urine samples were tested as received and also following the addition of glucose. Table 3 shows data on 2075 random urines from hospital patients tested with the glucose oxidase test and Benedict's qualitative test. It can be seen that 1423 urines were negative with both tests. Approximately one-fourth of all of the urines (571 out of 2075) gave trace reactions with Benedict's test but only 19 of these urines also gave a positive reaction with the glucose oxidase test. As with urines from healthy subjects the application of confirmatory tests to urines giving a trace reaction with Benedict's and a negative reaction with the glucose oxidase test showed that the reducing substance in these samples was not glucose. Fourteen

Table 3. Comparison of Tests on Random Hospital Urines

	Benedict's qualitative		
	Negative	*Trace*	*1+ or more*
Glucose oxidase test Negative	1423	552	14
Glucose oxidase test Positive	36	19	31

Table 4. Results of Blind Tests on Random Urines With and Without Added Glucose

Glucose Added	*Glucose oxidase test*		*Tablet copper reduction test*	
	Negative (%)	*Positive (%)*	*Negative (%)*	*Positive (%)*
0 (1925 urines)	98	2	99	1
0.1%–0.25% (845 urines)	3	97	18	82
0.35%–0.85% (776 urines)	1	99	0	100
1%–4% (964 urines)	0	100	0	100

of the urine samples were 1+ or more with Benedict's test but were negative with the glucose oxidase test. Two of these urines contained over 0.3% ascorbic acid which was a sufficient quantity to account for the positive test. In addition, among these urines were samples containing galactose, lactose, and fructose. There were 36 urine samples that gave positive reactions with the glucose oxidase test and negative reactions with Benedict's test. In these cases the amount of glucose present was quite small and not sufficient to give a positive Benedict's test. In these urines glucose was demonstrated by fermentation with baker's yeast with subsequent disappearance of the positive glucose oxidase test reaction and decrease in total Nelson-Somogyi reducing substance.

Evaluation

In order to assess the performance of the glucose oxidase test, samples of urine with or without added glucose were tested as unknowns by trained and untrained operators. Each day a few urines which gave negative reactions with the tablet copper reduction test and the glucose oxidase test were chosen. To each urine small amounts of concentrated aqueous glucose solutions were added to give several concentrations of glucose ranging from 0.1% to 4%. The samples were coded and given as unknowns

to from one to five operators. Each operator tested each sample with the glucose oxidase test and recorded his result. Some rotation of operators occurred on different days so that every unknown was not tested by each operator. A separate trained operator tested each sample with the tablet copper reduction test. Results of the comparison of the copper reduction with the enzyme test are shown in Table 4. It is evident that the two tests have comparable accuracy with negative urines. The 2 per cent error with the glucose oxidase test and 1% error with the tablet copper reduction test on the negative urines was due to the operator's obtaining trace reactions on urines which had been classified as negative. With amounts of added glucose between 0.1% and 0.25% the tablet copper reduction test was positive 82 per cent of the time. This figure depends on the exact amount of glucose added as well as on the specific urine to which it is added. The glucose oxidase test was positive 97 per cent of the time with amounts of added glucose between 0.1% and 0.25%. This demonstrates the high sensitivity of the glucose oxidase test. With amounts of added glucose of from 0.35% to 0.85% the glucose oxidase test was positive in 99 per cent of the cases. The 1 per cent of samples giving negative results had extremely high concentrations of ascorbic acid.

DISCUSSION

From the results of these studies it is apparent that the glucose oxidase test is a simple, accurate, specific, sensitive test for urine glucose. It has potential usefulness in diabetes detection screening programs, in routine urinalyses in hospitals and physicians' offices, in the differential diagnosis of diabetic coma from insulin shock or other coma, in regular urine testing on known diabetics by both patients and physician, and in the differentiation of glucosuria from other meliturias.

SUMMARY

A simple, specific, sensitive, and speedy test for glucose in urine has been described. This enzymatic test based on the activity of glucose oxidase and peroxidase is called *Clinistix*. Data are presented to show that the test has a high accuracy with both positive and negative specimens.

REFERENCES

1. Muller, D., *Biochem. Z.* **199**, 136 (1928).
2. Cook, M. H., Free, A. H., and Giordano, A. S., *Am. J. Med. Tech.* **19**, 283 (1953).
3. Keilin, D., and Hartree, E. F., *Biochem. J.* **42**, 221 (1948).
4. Hawk, P. B., Oser, B. L., and Summerson, W. H., *Practical Physiological Chemistry*, New York, Blakiston, 1954, p. 921.

Measurement of Uric Acid in Biologic Fluids Using an Ion-Exchange Separation

Bernard Shapiro, David Seligson, and Ralph Jessar

With the technical assistance of William Garfinkle and Dorothy Senesky

THE MAJOR METHODS CURRENTLY IN USE for the determination of uric acid in biologic fluids are the colorimetric (1–3), the titrimetric (4), and the enzymatic (5–7). All of these methods are performed directly on the fluid to be measured and are therefore subject to the effects of interfering substances present in these fluids. Since the colorimetric and titrimetric technics depend on the reducing ability of uric acid, they are nonspecific and are susceptible to many conditions which interfere with color development or iodometric titration (8, 9). In addition, intensely colored fluids, such as bile, cannot be studied by these technics. The enzymatic-spectrophotometric method is specific but, because of the presence of proteins, it is necessary to measure relatively small changes in absorption of ultraviolet light in the presence of very high total absorption. Initial precipitation of protein may coprecipitate a variable amount of uric acid (8).

The method to be described eliminates these difficulties by first isolating the uric acid from the biologic fluid to be studied. This is achieved by ion-exchange separation. The purified uric acid may then be measured either by the colorimetric or the enzymatic technics or simply by the

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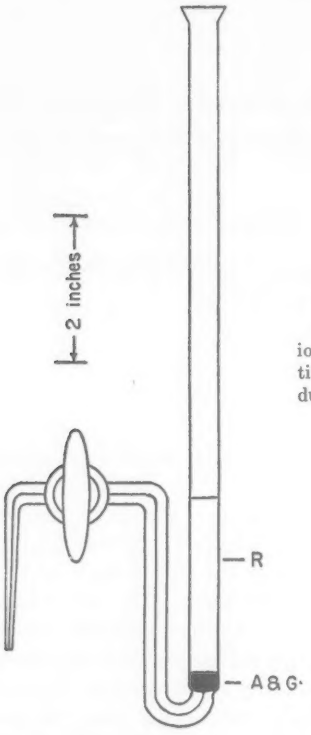

Fig. 1. Column used to hold ion-exchange resin for separation procedure. (*A & G*—alundum and glass wool; *R*—resin).

ultraviolet absorption of the uric acid eluate at 286 mμ. A similar approach has been reported by Bergmann and Dikstein (10) who have isolated uric acid by selective precipitation as the mercuric salt and have measured the absorbance of the redissolved uric acid at 290 mμ.

METHOD

Reagents

Dowex 2-x8,[1] 50 to 100 mesh, anion-exchange resin is prepared in a large batch as follows: 100 Gm. of resin is slurried into a large column, 5 cm. in diameter and 50 cm. long, and washed with 1000 ml. of 1N HCl. This is followed by distilled water until the eluate is nearly neutral. Then 1000 ml. of 1N NaOH is run through the column and followed by distilled water until the eluate is again nearly neutral. The final wash consists of

[1] Dow Chemical Company, Midland, Mich.

1000 ml. of 10 per cent NaCl and then distilled water until the eluate is free of chloride.

1N HCl, 1N NaOH, 10 per cent NaCl, 0.1N NaCl, 0.005N HCl.

Uric acid standard. Dissolve 10 mg. of uric acid, which has been purified on the anion-exchange column, in 150 ml. of hot water. Allow to cool and dilute to 200 ml. in a volumetric flask.

Apparatus

Small columns to hold ion-exchange resin (Fig. 1).

Beckman Model DU Spectrophotometer to measure absorbance at 286 mμ and a photoelectric colorimeter to measure absorbance near 660 mμ.

Procedure

Prepare a small column with alundum chips, glass wool, and 6 cm. of resin processed as described above. Add an appropriate amount of biologic fluid (containing about 50 to 200 μg. of uric acid) directly to this column. Adjust the flow of the column to 8 drops per minute. Wash the column with 10 ml. of distilled water, then with 10 ml. of 0.1N NaCl, and then with 10 ml. of distilled water again. Elute with 0.005N HCl. Uric acid from serum and urine will come off the column in the first 15 ml. of eluate and does not require fractionation. Other fluids may require fractionation to establish the location of the uric acid peak.

The quantity of uric acid can be determined by direct measurement of ultraviolet absorption at 286 mμ. Since there is a small column blank it is necessary to set up a standard curve initially by passing 0, 25, 50, 100, and 150 μg. of uric acid through separate columns. Measurements of uric acid in the eluate can also be made by the standard colorimetric technic; the Brown modification of the method of Folin (3), or by the following adaption of the enzymatic technic.

Enzymatic Technic

Add 5 ml. of eluate to each of two test tubes. To tube #1 add 2 drops of concentrated HCl and to tube #2 add 0.5 ml. of uricase[2] in glycine buffer (0.2 per cent uricase in 7.5 per cent glycine buffer at pH 9.4). After 2 hours at room temperature 0.5 ml. of enzyme in buffer is added to tube #1 and 2 drops of concentrated HCl is added to tube #2. The tubes are shaken immediately upon addition of the enzyme. The solution in tube #1 is read against that in tube #2 used as the reference or "blank" in the spectrophotometer at 286 mμ. The acid stops the enzyme reaction.

[2] Nutritional Biochemicals Corporation, Cleveland, Ohio, and Worthington Biochemical Corporation, Freehold, N. J.

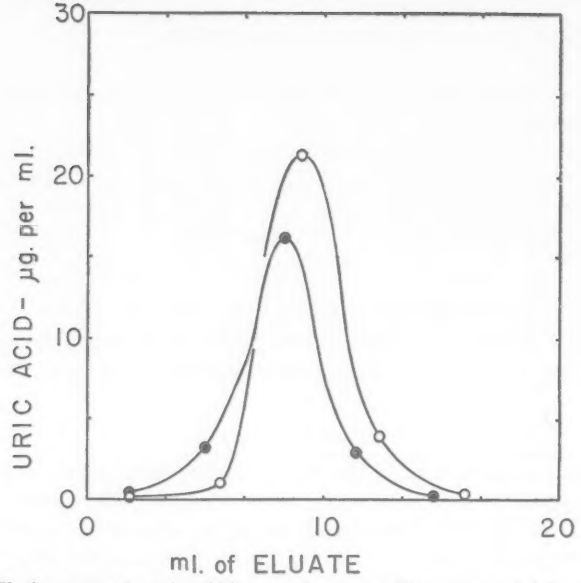

Fig. 2. Elution curves for uric acid in normal serum and in serum containing added uric acid. (●—normal serum, ○—serum with added uric acid).

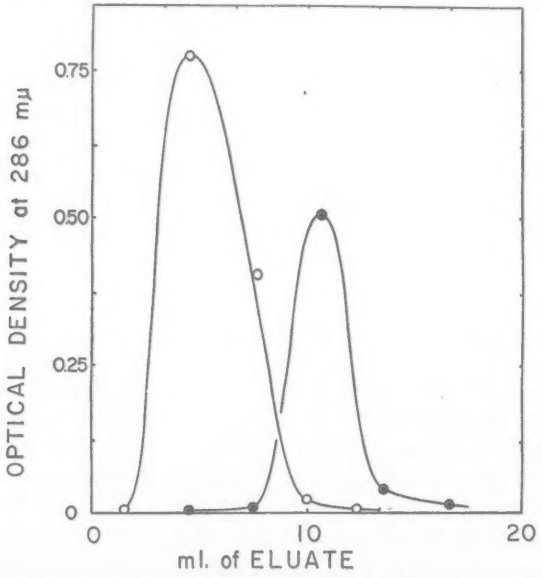

Fig. 3. Elution curves for uric acid in urine and bile. (●—bile, ○—urine).

RESULTS

The elution curves for uric acid in serum, serum with added uric acid, urine, and bile are shown in Figs. 2 and 3. Uric acid from serum and urine is easily eluted from the column in the first 15 ml. of eluate. The uric acid in the bile occasionally came off the column after the first 15 ml., and this may be dependent upon the high *p*H of bile.

Figure 4 shows the standard curve for different concentrations of uric acid placed on the column. The curve includes data observed on two different dates and on one date by the ultraviolet absorption and enzymatic technics. There is a small blank value, probably due to dissolved resin, which appears to be constant.

The ultraviolet absorption and enzyme values obtained on standard solutions before placing them on the resin column are slightly higher than values obtained on the eluates. This may indicate some small loss on the column or change in the structure of the molecule which might alter its response to these technics. The use of the standard curve corrects for this

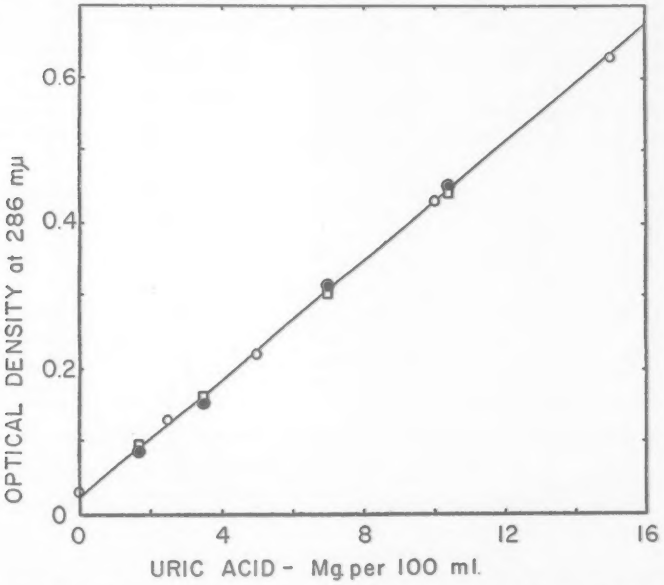

Fig. 4. Standard curve for different concentrations of uric acid placed on the ion-exchange column. (●—ultraviolet determinations on August 9, ○—ultraviolet determinations on July 13, □—enzymatic determinations on August 9). Fresh standard solutions were made for each date.

difference (Fig. 4). Recoveries from serum ranged from 89 per cent to 107 per cent, using the ultraviolet absorption method. The pre- and post-column values with standards studied by the Folin-Brown method were not significantly different.

Table 1. Determinations of Uric Acid in Serum, Urine, and Bile by the Direct Folin-Brown Method and on the Resin Eluates by the Ultraviolet Absorption, Folin-Brown, and Enzymatic Technics

Sample	*Direct Folin-Brown* (mg./100 ml.)	*Determinations on column eluate*		
		Ultraviolet (mg./100 ml.)	*Folin-Brown* (mg./100 ml.)	*Enzyme* (mg./100 ml.)
		URIC ACID IN SERUM		
42	4.2 ± 0.1	4.45 ± 0.06	4.35 ± 0.02	
92	5.3 ± 0.1	5.13 ± 0.07	5.80 ± 0.40	
95	3.8 ± 0.1	3.39 ± 0.01	4.85 ± 0.45	
20	5.5 ± 0.3	6.06 ± 0.14	6.64 ± 0.05	
25	3.2 ± 0.1	3.68 ± 0.13	4.30 ± 0.20	
83	6.2 ± 0.4	7.00 ± 0.10	6.50 ± 0.22	
86	11.4 ± 0.3	10.45 ± 0.15	10.34 ± 0.31	
40	4.5 ± 0.0	5.32 ± 0.08	5.19 ± 0.09	4.68 ± 0.00
50	5.4 ± 0.0	5.46 ± 0.06	5.40 ± 0.00	5.09 ± 0.11
78	5.5 ± 0.1	6.42 ± 0.08	5.41 ± 0.16	5.35 ± 0.25
80	9.3 ± 0.3	10.35 ± 0.00	9.56 ± 0.00	9.53 ± 0.13
87	6.8 ± 0.3	7.48 ± 0.13	6.45 ± 0.14	6.45 ± 0.15
27	5.6 ± 0.0	6.92 ± 0.07	5.92 ± 0.21	6.73 ± 0.33
28	3.0 ± 0.0	4.24 ± 0.06	3.29 ± 0.05	3.85 ± 0.05
		URIC ACID IN URINE		
BG23	146	95 6 ± 2.9	92.5 ± 1.5	88.5 ± 0.5
DS22	69	69.0 ± 0.0	62.0 ± 0.0	68.0 ± 2.0
BS23	168	115.8 ± 2.8	111.8 ± 1.8	106.5 ± 1.5
BG28	58	60.5 ± 3.3		54.5 ± 2.5
		URIC ACID IN T-TUBE BILE		
5-9		2.60 ± 0.05		2.08 ± 0.13
4-30		1.99 ± 0.12		1.37 ± 0.10
5-7		2.19 ± 0.01		1.56 ± 0.11
5-18		1.90 ± 0.10		1.19 ± 0.01

Table 2. Comparison of the Direct Folin-Brown Method on Serum with the Three Methods Applied to the Eluate

Direct serum Folin-Brown values compared with		*Mean of differences* (mg./100 ml.)	*Average of ratios*
Eluate values by	Ultraviolet absorption	0.5	0.92
	Folin-Brown method	0.3	0.93
	Enzymatic technique	0.2	0.96

The direct Folin-Brown values were subtracted from the eluate results or divided by the eluate results to obtain the differences or ratios respectively

Table 1 is a series of determinations of uric acid in serum, urine and bile giving the values obtained by the direct Folin-Brown colorimetric technic and the values obtained on the eluate by the ultraviolet absorption, Folin-Brown, and enzymatic methods. The values are given as the mean ± the difference between the duplicates and the mean. The ultraviolet values show the best comparison of duplicates and indicate the excellent reproducibility of results obtained using the ion-exchange resin columns. In Table 2 the values obtained on serums in Table 1 by the direct Folin-Brown method are compared with those obtained with each of the three technics applied to the eluate. The mean of the difference, between the direct Folin-Brown value for each serum and the values obtained by the other three methods, is given. The average of the ratios of the direct Folin-Brown value of each serum to each of the other three values is also given.

The enzymatic breakdown of uric acid by uricase is shown in Fig. 5.

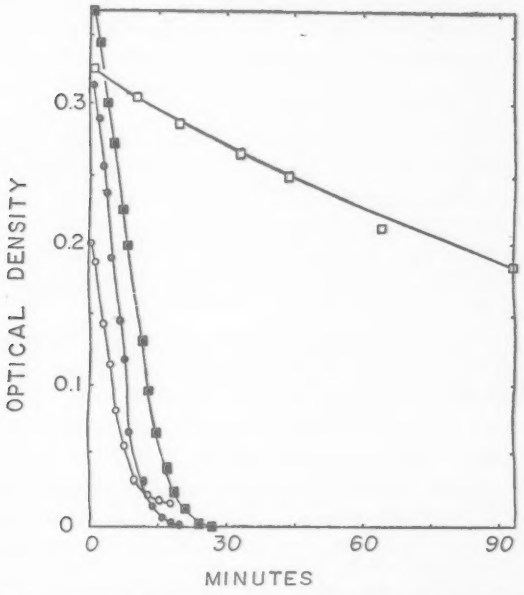

Fig. 5. The enzymatic breakdown of uric acid in eluate from serum and in standard solutions by uricase (●—uric acid in distilled water, ○—uric acid purified by ion exchange separation and dissolved in distilled water, ▣—uric acid in eluate from serum, □—uric acid in a stock solution containing formaldehyde).

At the concentration of enzyme used, the oxidation is very rapid and, with the amounts of uric acid ordinarily found in serum, is complete in 30 minutes. Two hours were allowed for safety. Also shown in this figure is the breakdown of a uric acid standard which was made up for the direct Folin-Brown method. The formaldehyde in this standard probably interfered with the enzyme action. The same stock uric acid powder dissolved in hot water gave a normal reaction.

The NaCl wash of the column before elution of uric acid yields a substance which absorbs in the ultraviolet light with a peak absorption of about 278 mμ and does not give color with the Folin-Brown technic. Urine contains this material, in higher concentration than in the serum. This compound has not been identified as yet, but it is definitely not adenine.

DISCUSSION

We have presented a new method for the determination of uric acid in biologic fluids. By trapping the uric acid on an ion-exchange column and then eluting it, we have isolated the substance so that the determination can be made by the relatively nonspecific ultraviolet absorption method. For serum and urine this technic is so simple that it can be used as a standard procedure in the clinical laboratory.

For greater specificity one can make the determinations on the eluate with the Folin-Brown or enzyme technics, although the mean difference between any two of the three methods applied to the eluate is less than 5 per cent. We consider the enzyme determination of uric acid in the eluate to be the most specific of our technics. It is especially applicable to the measurement of uric acid in biologic fluids which cannot be studied by direct technics.

The results of uric acid determination by the direct Folin-Brown technic in serum compare well with the results obtained by the new method. The mean difference between results is less than 10 per cent in comparing the direct Folin-Brown with the ultraviolet absorption method. The mean difference is less than 5 per cent in comparing it with the enzyme determination on the eluate. These data would seem to strongly support the reliability of the direct Folin-Brown technic in serum and contradict the disparity in results published on the direct Folin-Brown technic versus the direct enzyme technic (6, 9).

In the urine-uric acid studies, although the direct Folin-Brown values compared well with the new technics in some cases, there was a large discrepancy in others. This suggests the presence of interfering substances in some urines, and indicates the potential error of the Folin-Brown method for urine.

Bile values could not be checked by the direct Folin-Brown method. The enzyme technic on the bile eluates seems to give lower values than the ultraviolet technic. Uric acid in T-tube bile was much lower than that in one gallbladder bile which we studied, and which had a uric acid concentration of 24.4 mg. per 100 ml. Brugsch and Rother (11) in 1923 studied the uric acid in the bile from gallbladders of corpses. They used a copper precipitation technic and obtained uric acid concentrations ranging from a trace to 50 mg. per 100 ml. in 11 cases.

The ion-exchange separation technic presented here may also be used for the purification of uric acid and for the isolation and crystallization of uric acid from biologic fluids. Larger amounts of ion-exchange resin and larger columns may be necessary. By adding adequate amounts of uric acid-containing solution to the column, uric acid may be crystallized from the eluate by acidifying it with acetic or hydrochloric acid and allowing it to stand in the refrigerator overnight. This may be particularly useful in isotope experiments in which it is necessary to know the specific activity of the uric acid in a biologic fluid.

SUMMARY

A new method for the measurement of uric acid in biologic fluids is presented. It involves the isolation of uric acid by ion-exchange separation and quantitative determination of uric acid in the eluate by ultraviolet absorption, the Folin-Brown method or an enzymatic technic. The enzymatic determination on the eluate is the most specific of the technics.

A series of determinations of uric acid in serum compares well with determinations on the same samples using the direct Folin-Brown procedure. The direct Folin-Brown method appears to be in error in determinations on certain urine samples. Measurements of uric acid in bile are reported. The technic can be used in the routine clinical laboratory, and is particularly applicable to fluids which cannot be studied by direct methods.

REFERENCES

1. Benedict, S. R., *J. Biol. Chem.* **51**, 187 (1922).
2. Folin, O., *J. Biol. Chem.* **101**, 111 (1933).
3. Brown, H., *J. Biol. Chem.* **158**, 601 (1945).
4. Brøchner-Mortensen, K., *Acta med. Scand., Suppl. 84* (1937).
5. Praetorius, E., and Poulsen, H., *Scand. J. Clin. and Lab. Invest.* **5**, 273 (1953).
6. Feichtmeir, T. V., and Wrenn, T. W., *Am. J. Clin. Path.* **25**, 833 (1955).
7. Dubbs, C. A., Davis, F. W., and Adams, W. S., *J. Biol. Chem.* **218**, 497 (1956).
8. Natelson, S., *Standard Methods of Clinical Chemistry* **1**, 123 (1953).
9. Lous, P., and Sylvest, D., *Scand. J. Clin. and Lab. Invest.* **6**, 40 (1954).
10. Bergmann, F., and Dikstein, S., *J. Biol. Chem.* **211**, 149 (1954).
11. Brugsch, T., and Rother, J., *Klin. Wchnschr.* **2**, 1209 (1923).

Total Neutral 17-Ketosteroids

Clinical Method for Measurement

Marian S. Kafka and Philip K. Bondy

WITH INCREASING DEMAND for the determination of urinary total neutral 17-ketosteroids as a diagnostic tool, a need has developed for a simple, rapid method suitable for clinical use. Such a method was published by Drekter *et al.* (1) and modified by Masuda and Thuline (2). We have further modified the latter method to permit a larger number of samples to be run at one time. We have substituted equipment which is nonbreakable and less cumbersome, and reduced the number and delicacy of manual operations by making fewer transfers and by simplifying certain steps in the procedure.

MATERIALS AND EQUIPMENT

All chemical reagents are of analytical reagent grade.

1. Polyethylene screw-cap bottles of 200-ml. capacity.
2. Mechanical shaker.
3. Coleman Junior Spectrophotometer with modified adapter.
4. Concentrated HCl.
5. Constant temperature water bath.
6. Ethyl ether.
7. NaOH pellets.
8. Na_2SO_4, anhydrous.
9. Pyrex culture tubes, without lip, 15 x 85 mm.
10. Ethyl alcohol.

From the Department of Internal Medicine, Yale University, New Haven, Conn.

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11. Meta-dinitrobenzene.
12. Dehydroepiandrosterone. A standard solution containing 10 μg. per 0.2 ml., prepared in absolute ethyl alcohol.
13. Amyl acetate.

The Pyrex culture tubes are selected to fit the adapter of the Coleman Junior Spectrophotometer and calibrated with a dilute solution of methyl red to agree within ±0.02 optical density units.

Meta-dinitrobenzene is purified by dissolving 30 Gm. of the commercial reagent in a slight excess of ethyl alcohol by warming. When the *m*-dinitrobenzene is in solution, a pinch of Norite is added and the mixture filtered through a warm Buchner funnel containing filter paper covered with a layer of fine-milled asbestos. The filtrate is cooled until the *m*-dinitrobenzene has crystallized out. The crystals are dried, weighed, then further purified by the method of Callow *et al.* (3).

METHOD

Pipet 10 ml. of urine into a polyethylene bottle fitted with a screw-cap. Add 3 ml. of concentrated HCl and mix. With the cap screwed on lightly, incubate for 12 minutes at 85° in a water bath. Cool rapidly. Add 40 ml. of ethyl ether to the bottle, cap tightly, and shake for 10 minutes on a horizontal shaker (e.g., Eberbach, Ann Arbor, Mich.) in such a way that the aqueous and ether layers mix well but splashing is minimized. Aspirate off the urine (lower) layer. Add 20 ml. of 2.5N NaOH and shake for 5 minutes. Aspirate off the layer of NaOH. Add 20 ml. of distilled water and shake for 5 minutes. Aspirate off the aqueous layer, adding, when necessary, a little anhydrous sodium sulfate to remove traces of water. Detection of the interphases is facilitated if a strong lamp is placed behind the plastic bottle during aspiration. Pipet five 5-ml. aliquots of ether extract into the calibrated culture tubes. Three aliquots constitute triplicate samples of the unknown; two aliquots serve as pigment blanks. Pipet 0.2 ml. of dehydroepiandrosterone standard solution into each of two calibrated tubes. Evaporate the ether aliquots and standard tubes to dryness.

Prepare Solution A immediately before using by mixing two parts of 95% ethyl alcohol, three parts of 5.0 N NaOH (aqueous), and two parts of 2% alcoholic solution of *m*-dinitrobenzene. Solution B can be prepared once daily by mixing four parts of 95% ethyl alcohol with three parts of 5.0 N NaOH. To each of the dried urine pigment blanks add 0.7 ml. of Solution B and shake. To the dried samples and standards add 0.7 ml. of Solution A and shake well. Similarly, the reagent blank is prepared by adding 0.7 ml. of Solution A to an empty calibrated tube.

Incubate all the tubes in the dark at room temperature for 90 minutes. Just before this period ends, prepare Solution C by mixing one part of amyl acetate with one part of 75% ethyl alcohol. When the 90 minutes are over, add 2 ml. of Solution C to all tubes, stopper with foil-covered corks and shake vigorously. Put the test tubes in the dark for 8 minutes

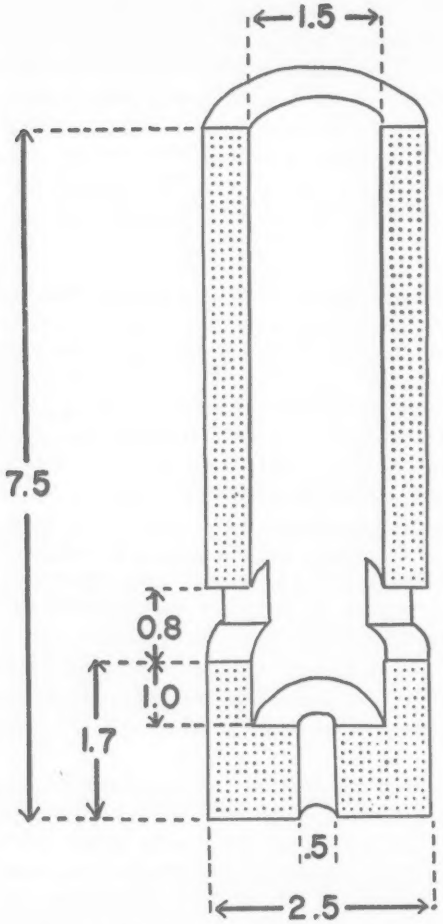

Fig. 1. Diagram of cuvet holder for Coleman Junior Spectrophotometer. The figure shows the front half of the holder cut away. The adapter can easily be made from 1″ birch dowel. Measurements are in centimeters.

and read at wavelength of 510 mμ on the Coleman Junior Spectrophotometer. The Coleman adapter must be modified so that the light beam passes through only the top (solvent) layer in the test tube. This can be accomplished by making a wooden adapter of the specifications given in Fig. 1.

CALCULATIONS

$$\frac{\text{Absorbance of sample} - \text{absorbance of pigment blank}}{\text{Absorbance of standard}} \times \frac{.01 \text{ mg. of KS (in standard)}}{\frac{5}{40} \times 10 \text{ ml.}}$$

× total urine volume in 24 hours = mg. neutral 17-ketosteroid in 24 hr. urine specimen. The dilution correction factor = .008; consequently, the equation can be simplified as follows:

$$\frac{OD_s - OD_b}{OD_{st}} \times .008 \times V_u = \text{mg./24 hr.}$$

EXPERIMENTS AND RESULTS

The duration of hydrolysis in our method is greater than that used by Masuda and Thuline, because the liquid inside the plastic bottles heats less rapidly than the same liquid contained in glass vessels.

When aliquots of four 24-hour urine specimens were run simultaneously in glass vessels and polyethylene bottles, the values obtained were in good agreement. For each specimen duplicate aliquots were run in glass and polyethylene. The results are given in Table 1.

The validity of premixing the Zimmermann reagents as described above was determined by comparing the intensity of color formed in duplicate aliquots of eight 24-hour urine specimens after adding the reagents one by one with that observed with the combined Solutions A and B. Table 2

Table 1. Comparison of Color Developed in Urines Hydrolyzed in Various Types of Vessels

Specimen	*Total 24-hr. vol.* (ml.)	*17-KS in 24-hr.*	
		Glass (mg.)	*Polyethylene* (mg.)
R	1300	8.3	8.7
K	850	11.5	11.5
I	445	8.9	8.3
C	615	5.0	4.8

shows the total number of milligrams of 17-ketosteroid obtained by the two types of treatment. The variation in the values obtained by the two different methods of adding Zimmermann reagents lies within the variation in the reproducibility found in our laboratory when running the same urine on different days. The temperature at which the Zimmermann color develops may vary between 25° and 35° without altering the intensity of

Table 2. Effect of Combining Zimmermann Reagents on the Intensity of Color Developed

Specimen	*Total 24-hr. vol.* (ml.)	*17-KS in 24 hr.*		
		Separate reagents (mg.)	*Combined reagents* (mg.)	*Difference* (mg.)
M	1350	16.9	17.7	0.8
C	1930	20.9	21.5	0.6
H	1210	15.5	15.0	0.5
B	1950	11.0	10.5	−0.5
W	1425	11.9	11.4	−0.5
O	890	9.5	8.9	−0.6
S	342	2.7	2.9	0.2
V	555	10.8	10.7	−0.1
			Mean ± s.e.:	−0.1 ± 0.6

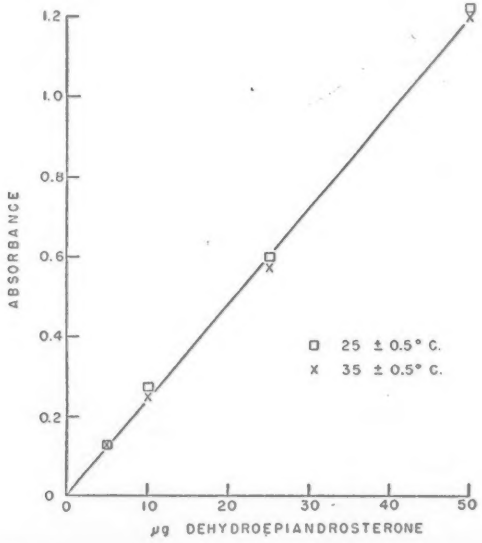

Fig. 2. Lack of influence of the temperature at which color development takes place. Note also close adherence to Beer's law.

the final color developed, at steroid concentrations between 5 and 50 μg. (Fig. 2).

Masuda and Thuline suggested that the standard steroid be added to portions of each urine extract as an "internal standard," but this proved clumsy and introduced a considerable potential error, since the absorbance of the standard was calculated from the difference between the readings. It proved simpler to analyze a pure steroid as a standard, and the results obtained were comparable to those obtained by the original method.

Sodium hydroxide was substituted for potassium hydroxide in the Zimmermann reaction because the color developed is a little more intense per unit steroid concentration, making for readings in a more desirable part of the spectrophotometer range in urines with low steroid concentrations.

The absorption spectrum of the Zimmermann color of urinary extracts is similar to that of pure dehydroepiandrosterone (Fig. 3).

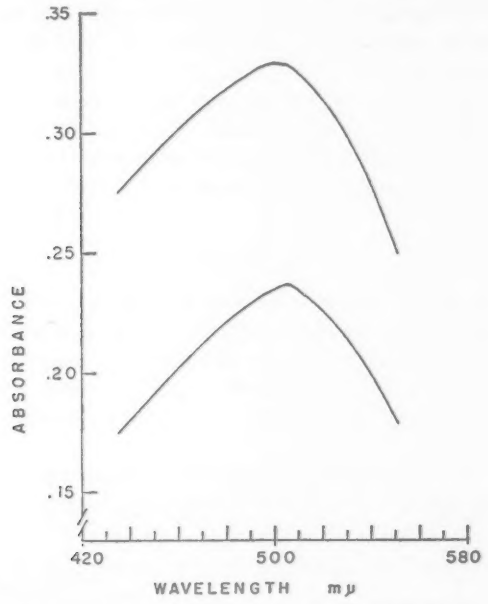

Fig. 3. Absorption spectra of urinary extract (*upper curve*) and of crystalline dehydroepiandrosterone (*lower curve*). The absorbance was measured in a Coleman Junior Spectrophotometer using 15 mm. cuvets.

SUMMARY

The Masuda and Thuline method for measuring urinary neutral 17-ketosteroids has been modified by substituting polyethylene bottles and a mechanical shaker for separatory funnels and manual shaking, and by developing the color and reading it in the same test tube. Combining Zimmermann reagents before addition eliminates the necessity for frequently pipeting small volumes. The new method permits the analysis of 10 to 15 urines in a single operation without sacrificing accuracy or specificity.

REFERENCES

1. Drekter, I. J., Pearson, S., Bartczak, E., and McGavack, T. H., *J. Clin. Endocrinol.* 7, 795 (1947).
2. Masuda, M., and Thuline, H. C., *J. Clin. Endocrinol. and Metabolism* 13, 581 (1953).
3. Callow, N. H., Callow, R. K., and Emmens, C. W., *Biochem. J.* 32, 1312 (1938).

Photometric Procedure for Determining Esterase Activity

Harold A. Ramsey

IN MOST OF THE ANALYTIC PROCEDURES for assaying the esterase content of biologic tissues and fluids, the extent of enzymatic hydrolysis is determined either titrimetrically, manometrically, or photometrically. In the photometric methods the alcoholic component of the substrate is such that its unesterified form can be measured either directly as such (1–6), or indirectly as a colored derivative (7–15). The introduction of photometry into the study of esterases marked an important advancement in the refinement of methods. Nevertheless, some of the photometric procedures possess undesirable features. Thus, the primary objective of the study reported herein was to devise an improved method for determining esterase activity.

EXPERIMENTAL

The technic of chromogenesis used most widely in the methods of indirect measurement is that of coupling the alcoholic component, when phenolic in nature, with a diazonium salt to form an azo dye. The present method employs esters of 2-naphthol as substrates. Chromogenesis is achieved by coupling the 2-naphthol with 4-sulfamoylbenzenediazonium chloride.

The stepwise procedure of the assay is presented in the ensuing section, which is followed by a discussion of some of the chemical and biologic aspects of the method.

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PROCEDURE

Reagents

1. Stock solution of 2-naphthyl ester, 2.96×10^{-2} M. To a 10-ml. volumetric flask, add successively 3.75 Gm. of melted Brij 35[1], 0.296 mM. of a 2-naphthyl ester and approximately 5 ml. of redistilled 1,4-dioxane, reagent grade. Mix the contents of the flask, warming slightly if necessary to obtain solution, and dilute to volume with additional dioxane. The ester should contain no free 2-naphthol or, at the most, only traces. Stock solutions of the caprylate and the palmitate esters are stable for at least several months if refrigerated.

2. Phosphate or citrate buffer, 0.067M. The choice of buffer will depend upon the *p*H optimum of the esterase.

3. Phosphate buffer, 1.0M, *p*H 6.8.

4. 4-Sulfamoylbenzenediazonium chloride solution (16). Prepare this reagent by mixing equal volumes of a solution of sulfanilamide, 0.035M in 0.48N hydrochloric acid, and a solution of sodium nitrite, 0.042M. Let this mixture stand for several minutes at room temperature, and then place it in an ice bath. When kept ice-cold, this reagent is stable for at least 6 to 8 hours.

5. Hydrochloric acid, 1.6N.

6. Sodium hydroxide, 3.3N.

Assay Technic

Prepare a buffered solution of substrate in the following manner immediately prior to its use. All reagents should be at room temperature. Into a gently agitated mixture of 40 ml. of 0.067M phosphate or citrate buffer and approximately 50 ml. of water, slowly add 1 ml. of the stock solution of ester with a pipet, the tip of which is held beneath the surface of the mixture. Dilute to 100 ml. with additional water.

Transfer 5 ml. of the buffered solution of substrate to a 25- x 200-mm. Pyrex test tube calibrated to contain 25 ml. Place the tube in a constant-temperature water bath. After equalization of temperature, add 1 ml. of an esterase preparation, and mix the contents of the tube thoroughly. Following incubation, the length of which is determined primarily by the rate at which the substrate is hydrolyzed, add 5 ml. of 1.0M phosphate buffer, *p*H 6.8. Immediately add 0.5 ml. of 4-sulfamoylbenzenediazonium chloride solution. Wait exactly 1 minute for the de-

[1] Brij 35, a polyoxyethylene lauryl alcohol, is a colorless, nonionic surfactant having a melting range of approximately 40–44° and is manufactured by Atlas Powder Company, Wilmington 99, Del.

velopment of color, and add 5 ml. of 1.6N hydrochloric acid. Place the tube in water which is kept at a rolling boil for 20 minutes. Cool the contents of the tube to room temperature. Add 5 ml. of 3.3N sodium hydroxide, letting it flow down the wall of the tube. Without undue agitation, dilute the contents of the tube to 25 ml. with water and mix thoroughly. Measure the optical density of this solution at 460 mμ using as a reference blank the solution from a concomitant control in which the esterase was inactivated prior to incubation by heating.

Many esterase preparations may be assayed simultaneously by staggering the addition of enzyme to the buffered solution of substrate at 2-minute intervals. In order that all samples will be incubated for the same length of time, the sequential addition of 1.0M phosphate buffer, 4-sulfamoylbenzenediazonium chloride solution and 1.6N hydrochloric acid, all of which requires approximately 1.5 minutes for a single tube, is staggered also at 2-minute intervals. At this stage, i.e., after addition of the hydrochloric acid, subsequent steps need not be performed immediately because the color is stable if the mixture is not exposed to sunlight.

Standard curve

From stock solutions of 2-naphthol in 1,4-dioxane and Brij 35, prepare buffered solutions of this compound in the same way that the buffered solution of substrate is prepared. Take 5-ml. samples of these buffered solutions, and, except for incubating them with esterase, subject them to the same procedure outlined in the assay technic. When measuring the optical densities of the final solutions, use as a reference blank the solution of a concomitant control without the 2-naphthol. The stock solutions of 2-naphthol are somewhat less stable under refrigeration than are those of the 2-naphthyl esters and, therefore, should be prepared immediately prior to their use. The standard curve is reproducible, thus obviating the preparation of standards for every series of assays.

DISCUSSION

2-Naphthyl caprylate and 2-naphthyl palmitate, the esters used in this study were synthesized according to the general procedure of Nachlas and Seligman (9)[2] and possessed melting points of 43.0–44.0° and 69.0–70.5°, respectively. The effect of *p*H on the nonenzymatic hydrolysis of these two esters was not studied specifically, but assays have been conducted over the *p*H range of 4.6 to 7.9 with either no or very little such hydrolysis occurring.

[2] Another method for the synthesis of 2-naphthyl esters has been described by Gomori (7).

Aqueous mixtures of water-insoluble esters frequently have been prepared by dissolving the ester in a water-soluble organic solvent and then introducing a small quantity of this solution into water (3, 7, 8, 9, 11, 14, 17). Such action with 2-naphthyl caprylate and 2-naphthyl palmitate, as well as many other esters, produces an opalescent suspension, which is undesirable for photometric measurement. In the present study it was found that nonopalescent solutions of substrate could be obtained if a sufficient quantity of Brij 35 were added to the mixture of ester and organic solvent. The suggested concentration of Brij 35 in the stock solution, 0.375 Gm. per ml., is in excess of the minimum required to produce nonopalescence. If the buffered solution of substrate is refrigerated, however, precipitation of the ester may occur.

Chromogenesis is accomplished by coupling 4-sulfamoylbenzenediazonium chloride with 2-naphthol to form 1-(4-sulfamoylbenzeneazo)-2-naphthol, an azo dye. The selection of this particular diazonium salt was based on its previous use in the quantitative determination of 2-naphthol by Harfenist and Baltzly, who were studying the acid-catalyzed alcoholysis of 2-naphthyl esters. Inasmuch as the reactants are the diazonium ion and the naphthoxide ion, not the undissociated naphthol molecule (18), the velocity of coupling increases as the hydrogen ion concentration of the reacting medium decreases. The purpose, therefore, of adding the 1.0M phosphate buffer having a *p*H of 6.8 immediately prior to the diazonium salt is to provide a hydrogen ion concentration that is low enough to facilitate rapid formation of the azo dye yet high enough to prevent nonenzymatic hydrolysis of the substrate. The procedure of Harfenist and Baltzly utilizes a solution of sodium acetate instead of a phosphate buffer. The phosphate buffer was selected for the assay described herein because of its greater buffering capacity in the region of *p*H 6.6–6.8. The quantity of phosphate buffer used, 5 ml., is sufficient to minimize *p*H changes that are induced either by the buffer employed during incubation or by the solution of diazonium salt. Dye formation, for which 1 minute is allotted, reaches completion between 15 and 30 seconds after the diazonium salt is added.

One half milliliter of the solution of diazonium salt is equivalent to six times the quantity of ester present in 5 ml. of the buffered solution of substrate. Thus, the excess diazonium salt must be destroyed to prevent it from coupling with any 2-naphthol that may be liberated subsequently by nonenzymatic hydrolysis, e.g., that caused by the addition of sodium hydroxide. Harfenist and Baltzly effected this destruction immediately after dye formation by heating the solution to its boiling point, which

action converts the excess diazonium salt to 4-hydroxybenzenesulfonamide (18), a colorless compound (19). In the present study such heating, even in the absence of 2-naphthol, produced an intense yellow color, which is believed to be an additional azo dye formed by some of the diazonium salt coupling with its own degradation product. This difficulty was circumvented by lowering the *p*H to approximately 1.4 with hydrochloric acid after formation of the naphthyl azo dye. The excess salt then can be destroyed by heating without encountering the undesirable side reaction because the coupling of diazonium salts with phenolic compounds does not occur in a strongly acid solution (18). The naphthyl azo dye is unaffected by such heating.

The naphthyl azo dye, although some of it may be held in suspension by the Brij 35, is insoluble in acid but is soluble in a strongly alkaline medium. Thus, sodium hydroxide is added, whereupon the dye changes in color from yellow-orange to red-orange. The color change, which suggests that the hydroxy radical of the dye undergoes ionization, requires approximately 4.5 ml. of 3.3N sodium hydroxide to complete. The relative optical density of the dye as a function of wavelength is illustrated in Fig. 1. The optical density at 460 mμ is directly proportional to the concentration of 2-naphthol over the range of 0 to 240 μg. per 25 ml. (Fig. 2). The stability of the dye is excellent provided that exposure to direct sunlight, which causes fading, is avoided. Solutions held under somewhat subdued lighting for as long as 24 hours decreased only slightly in optical density.

Inasmuch as several nonionic surfactants have been reported to reduce the activity of pancreatic lipase (20), investigation of the effect of Brij 35

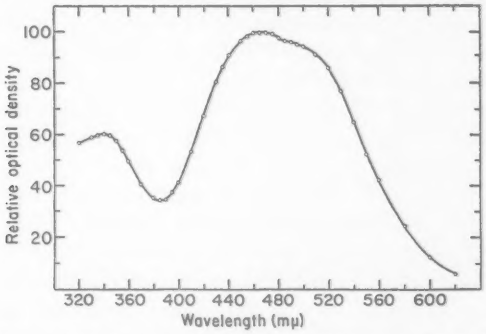

Fig. 1. Relative optical density of 1-(4-sulfamoylbenzeneazo)-2-naphthol as a function of wavelength.

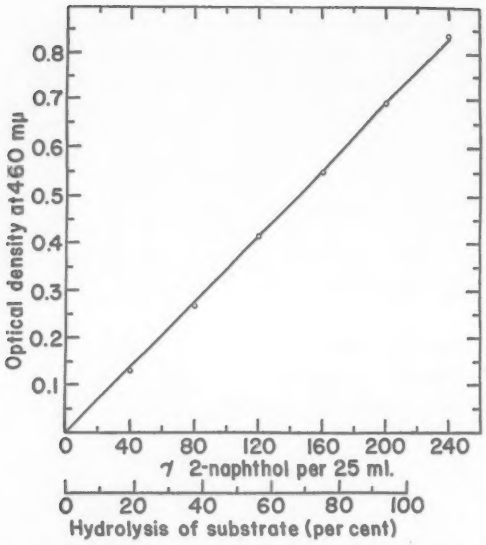

Fig. 2. Relationship between optical density at 460 mμ (Beckman D. U. spectrophotometer) and the concentration of 2-naphthol.

on the rate at which 2-naphthyl caprylate and 2-naphthyl palmitate are hydrolyzed by different esterases was deemed advisable. Stock solutions of ester were prepared such that the buffered solutions of substrate varied in concentration of Brij 35 (75 to 525 mg./100 ml.) but contained equal levels of all other components. The sources of esterase were aqueous extracts of the liver of swine and pharyngeal tissue of young calves. The results of these determinations are presented in Fig 3, in which the rates of hydrolysis are expressed in arbitrary units, the maxima being assigned the value of 100 and all others adjusted in proportion thereto. These data clearly indicate the Brij 35 may have either a stimulatory or an inhibitory effect on the rate of enzymatic hydrolysis. The concentration for maximum activity depends markedly on the substrate and, to a lesser extent, on the source of esterase. The rate of enzymatic hydrolysis also is affected by 1,4-dioxane. When present in the buffered solution of substrate at the level of 5 to 10 per cent, this solvent reduces esterase activity but does so to a lesser extent than the same concentration of acetone. Thus, 1,4-dioxane was selected for the procedure described herein, and its concentration was reduced to approximately that required to liquefy the stock solution of ester.

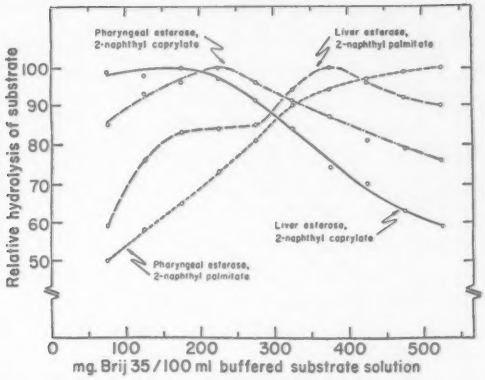

Fig. 3. Effect of Brij 35 on the relative hydrolysis of 2-naphthyl esters by pharyngeal esterase at *p*H 6.5 and by liver esterase at *p*H 7.45.

The effect of enzyme concentration on the rate at which 2-naphthyl caprylate is hydrolyzed is illustrated in Fig. 4. The source of esterase was the pharyngeal tissue mentioned previously. Only the initial rates of hydrolysis appear to be directly proportional to enzyme concentration.

This procedure is suited for assaying blood serum as well as tissue extracts. The results from such determinations are presented in Table 1. One milliliter of a fifty-fold dilution of serum was used per assay. Determinations were made in triplicate to illustrate the precision of the method. In one set of the triplicate samples, a flocculant precipitate was noted following the heating in boiling water. This precipitate, presumably protein, went into solution upon addition of the sodium hydroxide and caused no interference in the determination.

The procedure described is believed to have several advantages over certain other photometric esterase procedures. First, it does not require a photometer capable of measuring light absorption in the ultraviolet region of the spectrum as do the methods of Zeller and Hofstee (3–5). A second advantage is that the 2-naphthyl esters do not appear to undergo nonenzymatic hydrolysis as readily as the substrates reported in some methods (1–5, 12). The solubility of the naphthyl azo dye in a strongly alkaline medium also is advantageous in that it eliminates the necessity of having to extract the dye with an organic solvent (9–11) or of having to hold it in colloidal suspension with a surfactant (7). In other azo dye methods (7, 9–11), with the exception of one (14), no adjustment in *p*H is made immediately prior to dye formation, the rate of which is dependent upon

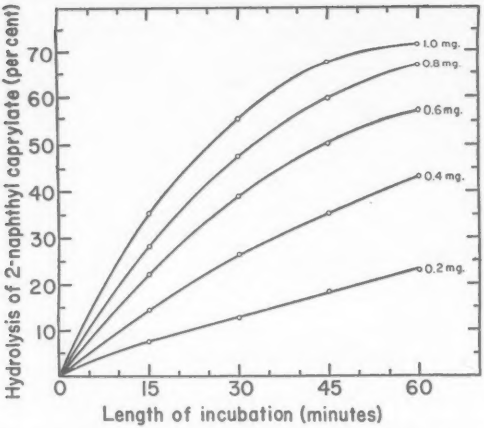

Fig. 4. Progressive hydrolysis of 2-naphthyl caprylate at *p*H 6.5 by the esterase from various quantities (0.2, 0.4, 0.6, 0.8, and 1.0 mg.) of an acetone powder of calf pharyngeal tissue.

Table 1. Hydrolysis of 2-Naphthyl Caprylate by the Esterase of Blood Serum at *p*H 7.0

Source of serum	*Age of animal*	*Triplicate determinations (% hydrolysis)*		
Calf	1½ weeks	0.8	0.6	0.7
Calf	1½ weeks	1.6	1.8	1.7
Calf	8 weeks	6.7	6.8	6.9
Calf	8 months	11.7	11.7	11.6
Calf	8 months	10.2	10.2	10.0
Calf	8 months	8.1	8.0	8.1
Lamb	6 months	17.9	17.7	18.0
Lamb	8 months	28.6	29.5	29.1
Lamb	9 months	24.6	25.0	24.8
Lamb	9 months	25.3	25.2	25.3

*p*H. Thus, the flexibility of these procedures (7, 9–11) is reduced in that the *p*H of incubation may be too low to permit complete formation of the dye. Such is not the case for the procedure described inasmuch as the *p*H is adjusted with 1.0M phosphate buffer. Nor is the *p*H of the final solution nearly so critical as it is in the method of Huggins and Lapides as emphasized by Dirks and Boyer. The use of Brij 35 to prepare non-opalescent mixtures of the substrate also is believed to be advantageous, particularly in view of the fact that many esters are very insoluble in water.

The manner in which Brij 35 modifies the rate of enzymatic hydrolysis, on the other hand, reduces somewhat the flexibility of this method for studying either the specificity of a single esterase for different substrates or the rates at which a single substrate is hydrolyzed by different esterases. Nevertheless, the importance of studying the influence of stabilizing agents on enzyme activity is emphasized by the variable effect of Brij 35. Two other factors also may limit the flexibility of the present method. The first of these is the presence of excessive quantities of protein or particulate matter in the enzyme extract causing opalescence or a precipitate in the final solution. Thus far, such difficulty has not been encountered, although efforts have been made to clarify the enzyme extracts as much as possible either by centrifugation, or by freezing and thawing as suggested by Gomori (7). The second problem is the natural occurrence of compounds in tissues and fluids that will couple with the diazonium salt to yield a colored derivative. To date such compounds have been found in excessive quantities only in rumen fluid.

SUMMARY

A procedure for determining the esterase content of biologic tissues and fluids has been presented. Fatty acid esters of 2-naphthol are employed as substrates. The 2-naphthol liberated by hydrolysis is coupled with a diazonium salt to form an azo dye, the concentration of which is estimated photometrically. Distinctive features of this method are (1) the use of Brij 35 to prepare nonopalescent solutions of substrate, (2) the adjustment of *p*H to ensure complete formation of the azo dye, and (3) the solubilizing of the azo dye by adding sodium hydroxide. This assay procedure is sensitive to very small amounts of esterase, and the stability of its color is excellent if proper precautions are taken.

REFERENCES

1. Zeller, E. A., *Arch. Biochem.* **61**, 231 (1956).
2. Huggins, C., and Lapides, J., *J. Biol. Chem.* **170**, 467 (1947).
3. Hofstee, B. H. J., *Science* **114**, 128 (1951).
4. Hofstee, B. H. J., *J. Biol. Chem.* **199**, 357 (1952).
5. Hofstee, B. H. J., *J. Biol. Chem.* **199**, 365 (1952).
6. Purr, A., *J. Am. Oil Chemists' Soc.* **29**, 409 (1952).
7. Gomori, G., *J. Lab. & Clin. Med.* **42**, 445 (1953).
8. Greenbank, G. R., and Wright, P. A., *J. Dairy Sci.* **33**, 396 (1950).
9. Nachlas, M. M., and Seligman, A. M., *J. Biol. Chem.* **181**, 343 (1949).
10. Seligman, A. M., Nachlas, M. M., and Mollomo, M. C., *Am. J. Physiol.* **159**, 337 (1949).
11. Seligman, A. M., and Nachlas, M. M., *J. Clin. Invest.* **29**, 31 (1950).
12. Forster, T. L., Jensen, C., and Plath, E., *J. Dairy Sci.* **38**, 764 (1955).

13. Aldridge, W. N., *Biochem. J.* **57**, 692 (1954).
14. Gomori, G., *J. Lab. & Clin. Med.* **34**, 275 (1949).
15. Mounter, L. A., and Whittaker, V. P., *Biochem. J.* **54**, 551 (1953).
16. Harfenist, M., and Baltzy, R., *J. Am. Chem. Soc.* **69**, 362 (1947).
17. Ramsey, H. A., Wise, G. H., and Tove, S. B., *J. Dairy Sci.* **39**, 1312 (1956).
18. Noller, C. R., *Chemistry of Organic Compounds*, Philadelphia, Saunders, 1951.
19. Kermack, W. O., Spragg, W. T., and Tebrich, W., *J. Chem. Soc.* 608 (1939).
20. Wills, E. D., *Biochem. J.* **60**, 529 (1955).
21. Dirks, B. M., and Boyer, P. D., *Cereal Chem.* **28**, 483 (1951).

Paper Electrophoresis of Serum Proteins

A Note

L. D. Mellor

ONE OF THE TECHNICAL PROBLEMS encountered in the electrophoretic analysis of serum proteins on filter paper is the difficulty of obtaining good separation of the various protein fractions while at the same time preserving sufficiently narrow bands to give readily interpreted patterns after staining with bromphenol blue or amido black.

A partial solution is to apply the serum as a narrow bar with a wire loop and then allow the buffer solution to flow along the paper strip by capillary action to compress the serum bar still further. We have found that the final clarity of the stained serum fractions pattern can be greatly improved by running the strips in our usual manner for 18 hours at 100 volts in a Durrum-type tank, and then reversing the polarity of the leads from the power supply and running the strips in the reverse direction for 5 minutes at 400 volts.

It is important that this second high-voltage treatment of the strips is not prolonged; some initial trials are advisable. If six or eight strips are run using the same serum, half of them removed before the second high-voltage treatment, and the whole series then dried and stained, the effect of the reversed run can be easily seen.

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the Clinical Chemist

THE RESPONSIBILITIES OF THE MEDICAL PROFESSION IN THE USE OF X-RAYS AND OTHER IONIZING RADIATION

Statement by the United Nations Scientific Committee on the Effects of Atomic Radiation

I. INTRODUCTION

1. The United Nations General Assembly, being aware of the problems in public health that are created by the developments of atomic energy, established a Scientific Committee on the Effects of Atomic Radiation. This Committee has considered that one of its most urgent tasks was to collect as much information as possible on the amount of radiation to which man is exposed today, and on the effects of this radiation. Since it has become evident that radiation due to diagnostic radiology and to radio-therapy constitutes a substantial proportion of the total radiation received by the human race, the Committee considers it desirable to draw attention to information that has been obtained on this subject.

2. Modern medicine has contributed to the control of many diseases and has substantially prolonged the span of human life. These results have depended in part on the use of radiation in the detection, diagnosis and treatment of disease. There are, however, few examples of scientific progress that are not attended by some disadvantages, however slight. It is desirable therefore to review objectively the possible present or future consequences of increased irradiation of populations which result from these medical applications of radiation.

II. GENERAL SURVEY OF THE IRRADIATION OF HUMAN BEINGS

3. Man has always been exposed to some irradiation from natural sources. To this has now been added, as a result of modern discoveries and the applications of ionizing radiation and radioactivity, certain forms of artificial irradiation.

4. *Natural irradiation* is due to:

(1) Cosmic radiation,

(2) "Background" gamma radiation from radioactive substances present locally in the earth, rock or building materials, and from disintegration products of radon in air,

(3) Radiations emitted from natural radioelements such as potassium 40, radium, radon and carbon 14 which are incorporated in the body.

5. The amount of this natural radiation varies with locality, but has been estimated as usually delivering between 70 and 170 mrems per year to the gonads. Of this total, the major contributions are of about 45 per cent from local gamma radiations, 30 per cent from cosmic rays and 20 per cent from body potassium 40.[1]

6. *Artificial irradiation* is derived from:

(1) The contamination of the environment, the atmosphere, or water by radioactive waste from atomic industries or from users of radioelements;
(2) The radioactive fallout, at greater or lesser distances from the source, or radioactivity resulting from the explosion of nuclear devices;
(3) The occupational exposure of certain groups of workers: medical practitioners, radiologists, dentists, nurses, atomic energy workers, uranium or thorium miners, and the industrial or scientific users of radiation generators or radioactive isotopes;
(4) The medical use of X-rays, other ionizing radiations and radioelements in the detection, diagnosis, investigation and treatment of human diseases;
(5) The use of certain devices which emit radiation, such as television receivers, watches with luminous dials, and the X-rays generators used for the purpose of fitting shoes.

7. The amount of artificial radiation must vary considerably in different countries and we have inadequate information as to the over-all significance of these factors. In certain countries where estimates have been made, it appears that the greatest gonad irradiation of the population is due to diagnostic radiological procedures, the amount from this source about equalling that from all natural sources in certain instances. The total present contribution from occupational exposure, from the products of atomic industries from radiotherapy and from the radiating devices mentioned above (paragraph 6, sub-paragraph 5)) is likely to be very considerably smaller. That from radioactive fallout to the gonads appears at present to be in the region of 1 per cent of the natural gonad irradiation in most areas.[2]

8. Both the magnitude and the significance of these various sources are under review by the Committee. Since medical irradiation accounts for a substantial if not the major proportion of all artificial exposure, it is important that its magnitude should be known accurately for different countries and circumstances. The possibility of making such an assessment depends upon the help of the medical profession, and particularly on the adequacy and availability of records kept by doctors, dentists and organizations responsible for the use of ionizing radiation.

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[1] From reports sent by India, Sweden, the United Kingdom and the United States of America.

[2] According to the reports sent by the United Kingdom and the United States of America.

III. RADIATION HAZARDS

9. The medical use of radiation is clearly of the utmost value in the prevention, diagnosis, investigation and treatment of human disease, but the possible effects of this irradiation of individuals require examination.

10. Generally speaking, the irradiation of living beings may produce radiobiological effects either on the irradiated individual himself or, through him, on his descendants; the former being termed somatic and the latter genetic effects. Somatic effects vary according to the different organs or tissues affected, and range from slight and reversible disturbances such as cutaneous erythema to the induction of leukaemia or of other malignant diseases. The possible reversibility of the somatic effects of radiation received in small doses or at low dose rates encourages the belief that there are permissible doses of radiation which will not cause completely irreversible or significant somatic damage. The threshold for occasional somatic damage may, however, prove to be low. In the case of genetic effects, on the other hand, there may be no threshold. These effects increase with a frequency corresponding to the total amount of radiation received by the germinal tissues, and in the great majority of cases, are adverse.

11. Many other factors complicate the interpretation of radiobiological effects. The differences between whole and partial body radiation, between a single exposure and continuous irradiation, or between the effects of different types of radiation are still imperfectly understood. Biological differences in the radiosensitivity of various tissues, or of the tissues of people of different age or sex, obviously influence the nature of radiation hazards. It is clear, however, that any radiation of gonads, and any substantial irradiation of other tissues, involve a chance of significant damage which requires assessment.

IV. GENERAL RECOMMENDATIONS REGARDING THE MEDICAL AND OCCUPATIONAL IRRADIATION OF HUMAN BEINGS

12. The Radiological Profession, through the International Commission on Radiological Protection,[3] has undertaken a valuable and responsible duty in defining maximum permissible limits of exposure for the main radiation hazards.

13. The establishment of these maximum permissible levels for those who are occupationally exposed to radiation depends on the view that there are doses which, in the light of our present knowledge, do not cause detectable somatic injury in the individual irradiated; and on the consideration that the number of individuals concerned is small enough for the genetic effects on the whole population to be negligible. For the gonads, or for irradiation of the whole body, the levels are such as to exclude doses greater than 0.3 rem in any week or 3.0 rem in any 13 weeks, or a sustained irradiation rate greater than 5 rem per year. These values imply that no total dose of over 50 rem will have been received

[3] See the report of the International Commission on Radiological Protection, published in the *British Journal of Radiology*—Supplement 6, of December 1954, in the *Journal Francais d'electro-radiologie*—No. 10, of October 1955, etc.

by the gonads by the age of 30, or of over 200 rem by the whole body by the age of 60, in any occupationally exposed person.[4]

As regards irradiation of the whole population, it is considered prudent to limit the average dose to germinal tissues from artificial sources to the order of magnitude of that received from all natural sources.

14. In considering the extent to which the population is irradiated for medical purposes, it is essentially the genetic hazard which is involved although it seems possible that in certain circumstances somatic injury may occur occasionally after low doses of radiation. Otherwise, the relevant dose is that indicating the mean gonad irradiation among the population as a whole up to the end of the average reproductive period.

15. The extent of such genetic irradiation from diagnostic procedures has been found to be equal to at least 100 per cent of all natural radiation in two countries,[5] and that from a third equalled at least 22 per cent of this figure.[6] Even before obtaining more exact values for these and other countries, it is clear that the exposure can be substantial in countries with extensive medical facilities, and that it is essential to consider any ways in which this exposure could be reduced without detriment to the existing or developing values of medical radiology.

16. The Committee is therefore anxious to obtain the help of radiologists in suggesting through appropriate governmental channels any methods by which this total exposure could be reduced and in estimating the amount of reduction that might be expected from any such methods. In particular it would be valuable to know how much the radiation to the gonads could be reduced.

(a) by improved design or shielding of equipment,
(b) by fuller training of any individuals using radiographic or fluoroscopic equipment,
(c) by any local shielding of the gonads that is practicable, especially during abdominal or pelvic examination,
(d) by the use of techniques involving radiography rather than fluoroscopy when full information can be obtained by this means,
(e) by improvement of administrative arrangements designed to obviate unnecessary repetition of identical examinations of the same subject,
(f) by a general study of certain medical conditions such as that of peptic ulcers, to identify the circumstances in which the establishment of a radiological diagnosis has or has not a definite influence upon the treatment or prognosis given.

VI. SUMMARY

1. The Scientific Committee on the Effects of Atomic Radiation established by the United Nations General Assembly accepts the view that the irradiation of

[4] See the report of the International Commission on Radiological Protection, published in the *British Journal of Radiology*—Supplement 6, of December 1954, in the *Journal Francais d'electro-radiologie*—No. 10, of October 1955, etc.

[5] Sweden, United States of America.

[6] United Kingdom.

human beings, and especially of their germinal tissue, has certain undesirable effects.

2. Information received so far indicates that, in certain countries (Sweden, United Kingdom, United States of America), by far the most important artificial source of such irradiation is the use of radiological methods of diagnosis and that this may be equal in importance to radiation from all natural sources. It is possible that such radiation may be having a significant genetic effect on the population as a whole.

3. The Committee is fully aware of the importance and value of the medical use of radiations but wishes to draw the attention of the medical profession to these facts and to the need for a more accurate estimate of the amount of exposure from this source. The help of the medical profession would be most valuable to make it possible to obtain fuller information on this subject.

4. The Committee would be particularly grateful for information through appropriate governmental channels on ways in which the medical irradiation of the population can be reduced without diminishing the true value of radiology in diagnosis or treatment.

BROCHURE ON BIOLOGICAL STAINS

A new 52-page brochure, *Biological Stains and Related Products for Microtechnic*, has just been published by Harleco as the most recent addition to the group of catalog supplements produced by this company. The brochure features complete lists of biological stains in crystalline as well as solution form, along with listing of the products known as Harleco Parstains, and finally a section devoted to histological and histochemical reagents. Copies of the publication are available upon request of Hartman-Leddon Company, 5821 Market St., Philadelphia 39, Pa.

BOOK REVIEWS

Analysis of Insecticides and Acaricides

F. A. GUNTHER and R. C. BLINN. New York, Interscience Publishers, 1955, 696 pp., $14.00

This volume, the sixth in the publisher's series of monographs on chemical analysis, deals with the assay of chemical compounds in widespread use today as insecticides. The use of these compounds presents the serious problem of their residual toxicity and has made very important the question of reliable analysis of these compounds.

The general part of the book deals with presampling considerations, sampling procedures, the analysis itself, and the interpretation of data, applied to the general problems of composition analysis of technical-grade material. The specific problems of field sampling for insecticidal residues have seldom found description in the chemical and analytical literature, and in these sections are offered important information for those concerned with agricultural analysis. The special part presents analytical procedures for determining approximately ninety insecticides and acaricides of contemporary interest.

The authors deal mainly with plant residues, as their interest lies in that direction, and the isolation procedures are straightforward and uncomplicated. They do not go into the isolation and analysis from animal tissue, urine, or blood, a failing which this reviewer finds common to most texts of this type. The effect of ingestion of certain compounds or drugs may not be due to the initial compound, but to some metabolite formed by the body in its eagerness to eliminate a foreign substance. With the knowledge of metabolites and their toxicity, the original compounds may be chemically altered as to provide the same results as an insecticide, but rendered harmless to larger animals ingesting the compound with their food.

Metropolitan Hospital, New York H. D. APPLETON

ABSTRACTS

Editor: ELLENMAE VIERGIVER. *Contributors:* JOSEPH S. ANNINO, GLADYS J. DOWNEY, CLYDE A. DUBBS, ALEX KAPLAN, MARGARET M. KASER, MIRIAM REINER, HERBERT THOMPSON

Practical considerations of diseases of porphyrin metabolism. F. W. Sunderman, Jr., and F. W. Sunderman (*Jefferson Medical College, Philadelphia, Pa.*).

The authors discuss in detail the current concepts of porphyrin metabolism and the detection and identification of the porphyrins in urine and feces. A new classification of porphyrins is suggested and illustrative cases are reported.—*Am. J. Clin. Pathol.* **25,** 1231 (1955). (H. E. T.)

Determination of blood urea nitrogen using a simple stabilizing reagent. H. V. Connerty, A. R. Briggs, and E. H. Eaton, Jr. (*Veterans Administration Hospital, Wilmington, Del.*).

In the determination of urea nitrogen by the method of Karr [*J. Lab. Clin. Med.* **9,** 329 (1924)] the color can be stabilized and the development of turbidity prevented by the addition of one drop of 2% aqueous iodine solution to either the diluted blood filtrate or to the Nessler's reagent before using.—*Am. J. Clin. Pathol.* **25,** 1321 (1955). (H. E. T.)

A simplified paper chromatographic method for separation of glucose and fructose in urine. A. S. Cohen and N. Kantor (*Boston City Hospital, Boston, Mass.*).

Glucose and fructose in 0.02 ml. of urine from diabetic patients can be identified and separated by using a solvent system of *n*-propyl alcohol and water in a 3:1 ratio. The solvent travels about 14 cm. in 4 hours on Whatman #1 filter paper by the ascending technic. Although the glucose and fructose spots are not completely separated in this short period of time, each can be identified by spraying with a solution of naphthoresorcinol (0.2% in ethyl alcohol plus 0.1 volume of concentrated phosphoric acid) and drying at 90° for 5 to 10 minutes. Fructose appears as a distinct red-purple spot and glucose as a blue spot. Complete separation of the two hexoses can be effected by an overnight chromatogram. Semiquantitative estimations can be made by comparing the densities of these colors with those obtained with standard glucose and fructose solutions.—*Am. J. Clin. Pathol.* **25,** 1328 (1955). (H. E. T.)

Studies on phenylketonuria: IV. Serum proteins in phenylketonuria. D. M. Brown, M. D. Armstrong, and E. L. Smith (*University of Utah College of Medicine, Salt Lake City, Utah*).

The serum proteins of 18 individuals, age 8 months to 48 years, with phenylketonuria were studied to determine whether their inability to metabolize phenylalanine in a normal manner was associated with any evidence of a derangement in protein metabolism. Determinations were made in a Tiselius apparatus in Veronal buffer, 0.1M, and a *p*H of 8.6 and the descending boundaries were examined. The total protein was estimated by multiplying the nitrogen values obtained by Kjeldahl analysis by the factor 6.7. Serum phenylalanine was determined by the method of Kapeller-Adler [*Biochem. Z.* **252,** 185 (1932)]. The only significant abnormality was the occurrence of spike anomalies in the β-globulins of 8 subjects. These abnormalities, which were not correlated with the degree of mental impairment, disappeared when low phenylalanine diets were fed and the blood phenylalanine levels were reduced to the normal range. It is thought that the abnormal globulins may indicate some slight degree of liver impairment.—*Proc. Soc. Exptl. Biol. Med.* **89,** 367 (1955). (M. M. K.)

Competitive binding of calcium and magnesium with serum albumin. C. W. Carr (*University of Minnesota, Minneapolis, Minn.*).

Known quantities of calcium and magnesium chloride solutions were added to a solution of bovine serum albumin which had been electrodialyzed and adjusted to *p*H 7.4 with NaOH just prior to use. The resulting solutions were placed inside cellophane dialyzing membranes which were surrounded by an equal volume of $CaCl_2$-$MgCl_2$ solution and the system was allowed to equilibrate for 72 hours at 3°. The two compartments were then analyzed for their respective constituents. The results show that Ca and Mg compete with each other on an equal basis for combination with serum albumin. The ratio of the concentrations of bound Ca and bound Mg was equal to the ratio of the concentrations of ionized Ca and ionized Mg. The physiologic significance of the results is discussed.—*Proc. Soc. Exptl. Biol.* **80,** 546 (1955). (M. M. K.)

Isolation and determination of glycoproteins in cerebrospinal fluid. E. Roboz, J. B. Murphy, W. C. Hess, and F. M. Forster (*Georgetown University School of Medicine, Washington, D. C.*).

A procedure is described for the isolation of the glycoproteins from spinal fluid that also separates the lipids and free sugars. The carbohydrates in the glycoproteins were identified, after hydrolysis, by paper chromatography as mannose, galactose, fucose, and glucosamine. Methods also are given for the quantitative determination of hexose and hexosamine associated with the glycoproteins and the γ-globulins, and also for the neuraminic acid content. An increase in γ-globulin hexose was found in multiple sclerosis. The ratio of γ-globulin hexose

to glycoprotein was also increased but the ratio was not different from that found in some nondemyelinating diseases, which suggests that the hexose increase merely reflected the increase in γ-globulin.—*Proc. Soc. Exptl. Biol. Med.* **89,** 691 (1955). (M. M. K.)

Influence of pyridoxine on transaminase activity of human placenta, maternal and fetal blood. M. B. Glendening, A. M. Cohen, and E. W. Page (*University of California School of Medicine, San Francisco, Calif.*).

The glutamic-aspartic transaminase activity of whole blood and of homogenized placentas was determined by a modification of the method of Tonhazy, White, and Umbreit [*Arch. Biochem.* **28,** 36 (1950)]. The activity of blood was expressed as the number of μl. of oxalacetate formed per hour per ml. of blood and was calculated from the amount of pyruvate obtained after incubation of 0.1 ml. of blood for 10 minutes.

No difference was found in the transaminase activity of blood drawn from 18 pregnant women at the time of delivery and blood from 10 nonpregnant women. However, the addition of 10 mg. of pyridoxine hydrochloride daily to the diet of 6 pregnant women increased the blood transaminase activity to a mean value of 984 units, as compared with 748 units for the nonsupplemented group of pregnant women. In a single case 150 mg. of pyridoxine hydrochloride per day caused an increase of 81 per cent in transaminase activity on the third day and 120 per cent 32 hours later. Ten cord bloods had a mean value of 1467 units, the activity being concentrated in the red cells. Supplementation had no effect on the transaminase activity of cord blood or the placentas. The data are interpreted as indicating that the fetus has an adequate supply of pyridoxine for maximum transaminase activity, perhaps at the mother's expense, and that supplementation of the maternal diet with pyridoxine during pregnancy is desirable.—*Proc. Soc. Exptl. Biol. Med.* **90,** 25 (1955). (M. M. K.)

Colorimetric estimation of ultramicro quantities of calcium in human serum as the complex with alizarin. S. Natelson and R. Penniall (*Rockford Memorial Hospital, Rockford, Ill.*).

A rapid colorimetric method suitable for the routine clinical laboratory is described for estimating calcium. To a 15-ml. screw-cap tube are added 1.0 ml. of distilled water, 0.02 ml. of serum, 2.0 ml. of 1.0N triethanolamine, and 3.0 ml. of alizarin reagent (40 mg. recrystallized alizarin in 1 l. of *n*-octyl alcohol). The mixture is shaken for 20 minutes and centrifuged for 5 minutes. Two milliliters of the octyl alcohol phase, containing the Ca-alizarin color complex, are recentrifuged to assure clarity and the absorbance is measured at 560 mμ.

A constant correlation of 7 per cent is subtracted for magnesium interference, which may result in a possible error of −2 to +5 per cent for the Ca concentration. Therefore, if sample size permits and speed is not essential, the authors prefer a preliminary oxalate precipitation.

Absorption spectra, standard curves, recovery data, and correlation with the method of Clark and Collip are given.—*Anal. Chem.* **27,** 434 (1955). (C. D.)

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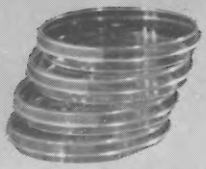

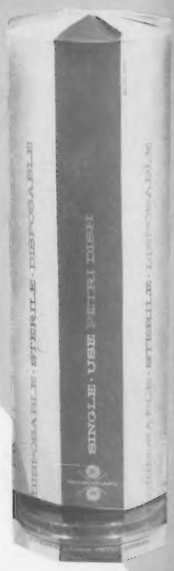

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